

THE EFFECTS OF ELAFIN GENE AUGMENTATION
ON ACUTE PULMONARY INFLAMMATION

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DECLARATION

The work described in this thesis was performed by me, except where otherwise acknowledged. No part of my work has been submitted in candidature for another degree.

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ABSTRACT

Several inflammatory lung disorders are characterised by airway neutrophilia and release of cytotoxic neutrophil products. In particular, human neutrophil elastase (HNE) is found in airway secretions from patients with emphysema, acute respiratory distress syndrome, cystic fibrosis and pneumonia. Inhibition of HNE is therefore a desirable therapeutic goal. Elafin is produced in the human lung and is a cationic, low molecular weight inhibitor of HNE. The hypothesis driving this work was that genetic augmentation of elafin may protect the lung against acute inflammatory injury. A replication-deficient adenovirus encoding human elafin cDNA under the control of the powerful murine cytomegalovirus promoter (Ad-elafin) was used to produce elafin augmentation in view of the natural tropism of adenovirus for respiratory epithelium.

Ad-elafin significantly protected pulmonary epithelial cells against the effects of both HNE and whole activated human neutrophils *in vitro*. These findings were extended by studying the effect of Ad-elafin on pulmonary neutrophilia induced by lipopolysaccharide (LPS) in mice. Intratracheal (IT) Ad-elafin, administered in doses low enough to obviate overt vector-induced inflammation, significantly augmented LPS-mediated neutrophilia. In addition, LPS significantly up-regulated elafin secretion from Ad-elafin transfected murine airways and from Ad-elafin transfected human pulmonary epithelial cells.

The demonstration of a cytoprotective effect for a low molecular weight, cationic elastase inhibitor capable of augmenting neutrophil recruitment during inflammation suggested a potential antimicrobial function for elafin. Elafin was shown to have significant antimicrobial activity against the respiratory pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*. On the basis of these observations, the hypothesis that elafin may be protective against inflammatory injury was tested *in vivo*. Low dose IT Ad-elafin (3×10^7 plaque forming units) was associated with a significant reduction in acute lung injury induced by *Pseudomonas aeruginosa* in mice.

These findings suggest that augmentation of endogenous host defence molecules can protect the lung against acute inflammatory injury. They further suggest that adenoviral constructs containing selective promoters may allow inflammation-specific expression of transgene using low doses of vector.

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ABBREVIATIONS

AAV	Adeno-associated virus
Ad	Adenovirus
Ad-elafin	Adenovirus encoding human elafin cDNA downstream of the murine cytomegalovirus promoter
Ad-lacZ	Adenovirus encoding the lacZ gene downstream of the murine cytomegalovirus promoter
α_1 -PI	Alpha ₁ -protease inhibitor
α_2 -M	Alpha ₂ -macroglobulin
APTT	Activated partial thromboplastin time
ARDS	Adult respiratory distress syndrome
BALF	Bronchoalveolar lavage fluid
bp	Base pairs
BPI	Bactericidal/permeability-increasing protein
BSA	Bovine serum albumin
C1705	<i>Staphylococcus aureus</i> strain C1705
CAR	Coxsackie-adenovirus receptor
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
cfu	Colony forming units
COPD	Chronic obstructive pulmonary disease
Da	Dalton
DAB	3,3'-diaminobenzidine tetrachloride
DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene diamine tetraacetic acid
EIA	Elastase inhibitory activity
ELISA	Enzyme-linked immunosorbent assay
FEV ₁	Forced expiratory volume in 1 second
fMLP	Formyl methionyl leucyl phenylalanine
FCS	Foetal calf serum
HIAS	Heat inactivated autologous serum

HIV	Human immunodeficiency virus
HNE	Human neutrophil elastase
HRP	Horseradish peroxidase
HSA	Human serum albumin
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Intraperitoneal(ly)
IT	Intratracheal(ly)
IV	Intravenous(ly)
kb	Kilobases
kDa	Kilodaltons
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
LT	Leukotriene
M	Molar
mCMV	Murine cytomegalovirus
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
mKC	Murine keratinocyte-derived chemokine
moi	Multiplicity of infection
MPO	Myeloperoxidase
mu	Map units
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NF	Nuclear factor
NGS	Normal goat serum
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PAF	Platelet activating factor
PAO1	<i>Pseudomonas aeruginosa</i> , strain PAO1
PBMC	Peripheral blood-derived mononuclear cells
PBS	Phosphate buffered saline
pfu	Plaque forming units
PIA	Pseudomonas Isolation Agar

PMA	Phorbol myristate acetate
PPE	Porcine pancreatic elastase
PRF-DMEM	Phenol red-free Dulbecco's Modified Eagles Medium
PRP	Platelet rich plasma
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcriptase polymerase chain reaction
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SLPI	Secretory leukocyte protease inhibitor
TBS	Tris buffered saline
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TSB	Tryptone soya broth
VEGF	Vascular endothelial growth factor
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

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CHAPTER 1

INTRODUCTION

1.1. OVERVIEW

Lung tissue may be damaged during episodes of pulmonary inflammation. Tissue injury implies that endogenous local defence mechanisms have been subverted, and that potentially destructive moieties such as human neutrophil elastase (HNE) function relatively unopposed. The work described in this thesis was stimulated by two fundamental questions. Firstly, can augmentation of host defence molecules such as elafin (an endogenous pulmonary inhibitor of HNE) protect lung tissue against the injurious effects of inflammation? Secondly, can augmentation of elafin be efficiently achieved using transfer of the elafin gene to those cells specifically susceptible to inflammatory injury?

Against this background, the following sections will outline the importance of the neutrophil in inflammatory processes. The ability of molecules derived from neutrophils to damage host tissue will be discussed, with emphasis on the importance of HNE. Endogenous inhibitors of HNE will be described, with particular reference to the function of elafin. Strategies available to augment host defence molecules in the lung will focus on gene transfer techniques, and in particular the use of recombinant adenoviral vectors.

1.2. ACUTE INFLAMMATION

Acute inflammation describes the sequence of programmed events occurring in living tissues exposed to noxious stimuli. In simple terms, the early stages of acute inflammation are characterised by vasodilatation, exudation of protein-rich fluid into tissue spaces, and the extravasation of circulating leukocytes, predominantly in the form of neutrophils (Figure 1). The noxious stimulus (eg invading bacteria) provokes host cells into the generation of a complex array of inflammatory and anti-inflammatory mediators, with the aim of eradicating the source of inflammation whilst restoring tissue viability. Acute inflammation is thought to have evolved to counter the effects of pathogenic microbes (reviewed by Weissman, 1992), and in this context the inflammatory process serves to isolate the offending micro-organism

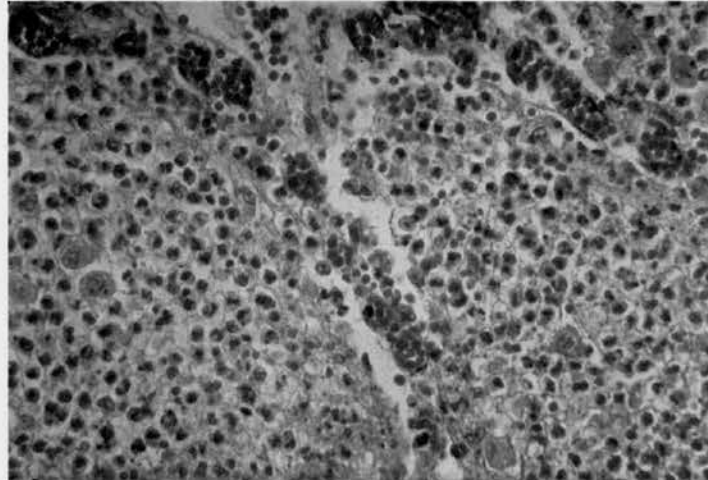


Figure 1. The histological features of acute pulmonary inflammation.

This section is from a patient with severe bacterial pneumonia. Alveoli are packed with proteinaceous exudate and extravasated neutrophils.

The section was stained with haematoxylin and eosin (original magnification x 200).

This slide was kindly provided by Dr Donald Salter, Department of Pathology, University of Edinburgh.

and to recruit circulating neutrophils which recognise, ingest and destroy bacteria. Removal of the noxious stimulus usually results in resolution of acute inflammation without detriment to the host, and the process can thus be regarded as a highly evolved and conserved mechanism of tissue protection.

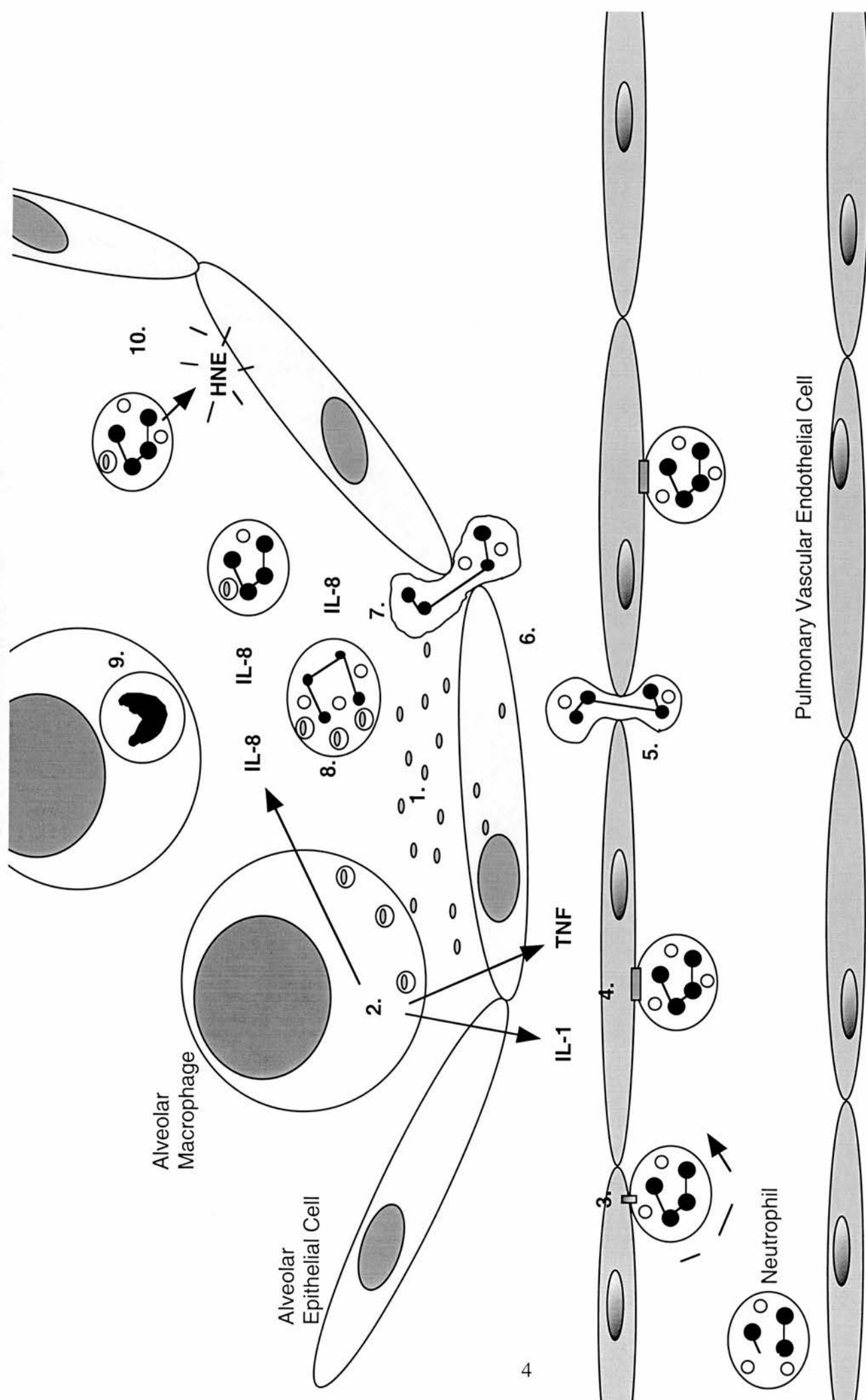
However, under particular circumstances acute inflammation can proceed to significant tissue injury. This may result through persistence of the noxious stimulus, and/or through the toxic effects of inflammatory mediators released by the host. As will be discussed in section 1.3.2., several potent cytotoxic agents are produced by neutrophils, and these may be released extracellularly under specific conditions. In this setting, it is notable that a highly developed network of "defence molecules" are elaborated by tissues in order to preserve integrity. A fine but complex dividing line therefore appears to exist between acute inflammation representing a beneficial adaptive response, and becoming an injurious maladaptive principle. Although a multitude of host factors may influence the outcome of acute inflammation, the central host defence cell involved is the neutrophil. A schematic representation of acute inflammation, with particular reference to the role of neutrophils, is shown in Figure 2.

It should be stressed from the outset that while "allergic inflammation" (which probably evolved in response to helminthic infestation and in which the eosinophil plays an important part (Gleich et al., 1992)) is of central importance to selected human diseases, the allergic response is not considered further in this thesis.

1.3. THE NEUTROPHIL

1.3.1. Origin and basic morphology.

The neutrophil is produced in bone marrow and is of myeloid lineage (reviewed by Murphy, 1976). A small proportion of the bone marrow pool of neutrophils is released into the circulation during health, such that a healthy human adult will have between 2×10^9 and 7.5×10^9 circulating neutrophils per litre of blood. The circulating neutrophil is thought to have a short half-life of only a few hours (Athens et al., 1961). The capacity for bone marrow to produce neutrophils is huge however, and the number of circulating neutrophils rises dramatically and acutely in response to inflammatory stimuli (Fliedner et al., 1964).



The circulating neutrophil is a highly differentiated cell, approximately 10-15 μm in diameter, characterised by the presence of a multilobed nucleus (reviewed by Murphy, 1976). The cytoplasm contains numerous granules, the predominant species being azurophilic (or primary) and specific (or secondary) granules, though less well characterised granules also exist.

1.3.2. The physiological function of neutrophils.

The neutrophil is thought to exist primarily to eradicate bacteria. This function was elucidated over one hundred years ago, and is beautifully described in the works of Metchnikoff (1887, 1905) and his colleague Gengou (1901). The principal functions of the neutrophil can be conveniently identified in the stages of the cell's passage from the circulation to the location of pathogenic bacteria. The neutrophil must "sense" an inflammatory stimulus in the microcirculation of a given tissue, migrate from the vascular space to the bacterial focus, then engulf and destroy bacteria. Failure of these mechanisms, as manifest in rare hereditary disorders, results in a propensity to recurrent infection (reviewed by Malech and Nauseef, 1997). Finally the neutrophil must be cleared from the site of inflammation without release of intracellular toxins. This sequence of events is summarised in Figure 2 and described in more detail below. Further information on the function of neutrophils can be obtained from a number of excellent review articles and monographs (Metchnikoff, 1905; Murphy, 1976; Lehrer et al., 1988; Haslett et al., 1989; Weiss, 1989; Windsor et al., 1993; Rossi and Hellewell, 1994; Albert, 1995; Shanley et al., 1995; Malech and Nauseef, 1997). In the context of the experimental work which follows it is worth noting that neutrophilic inflammation can be induced in tissues by local administration of lipopolysaccharide (LPS) (Hudson et al., 1977; Cybulsky et al., 1988), a product of Gram-negative bacteria important in the pathogenesis of septic shock (Morrison and Ryan, 1987). Indeed LPS is used specifically in order to effect pulmonary neutrophilia in Chapter 4. The biology of LPS and its role in initiating inflammation is outlined in Figure 3.

Circulating neutrophils are capable of forming reversible adhesive contacts with endothelial cells in post-capillary venules. These contacts are mediated by the interaction between a family of molecules known as selectins, and cell-surface carbohydrate ligands (Bevilacqua and Nelson, 1993). Expression of the ligands involved in mediating contact appears to be up-regulated by local inflammatory stimuli. Thus, L-selectin is constitutively produced by neutrophils and binds to

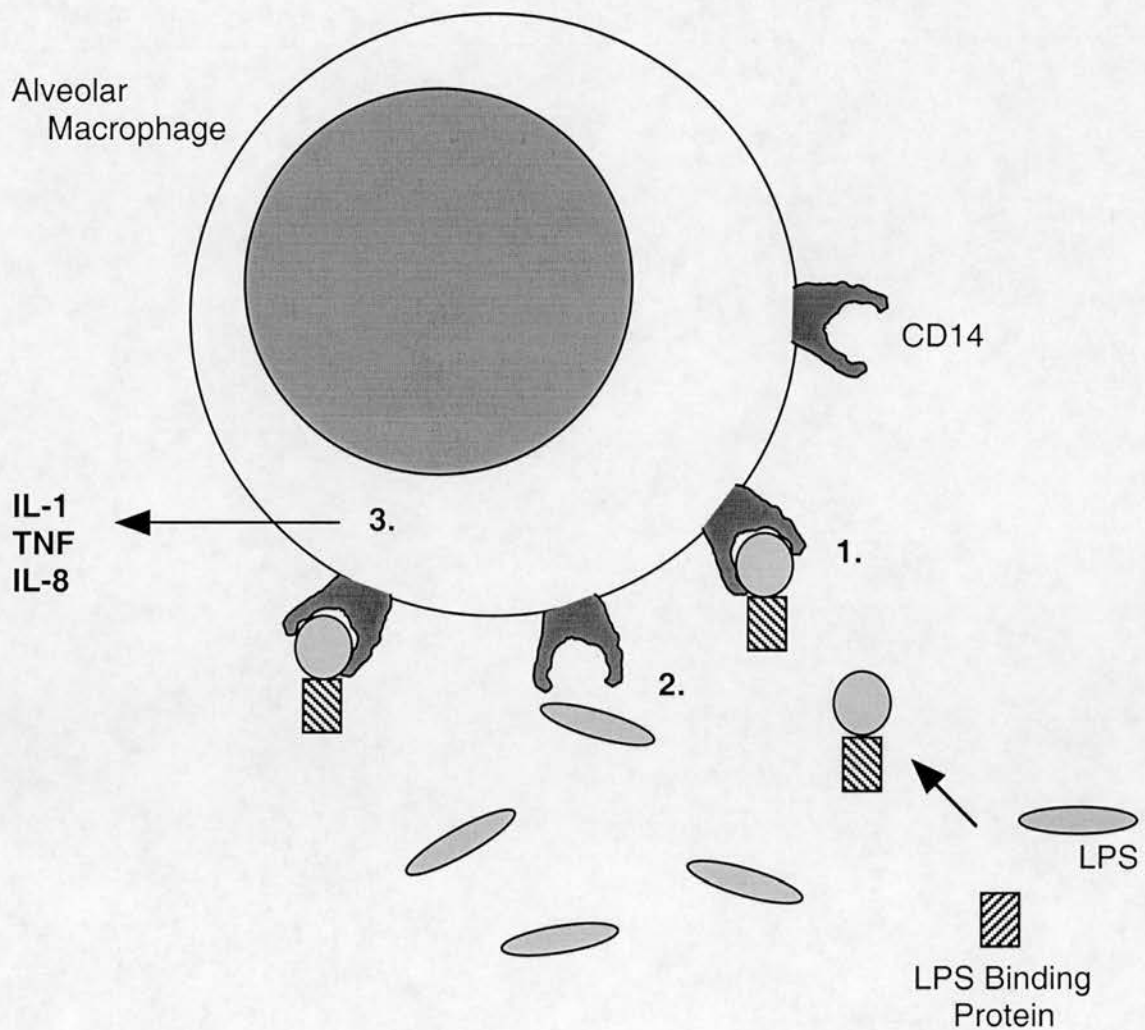


Figure 3. LPS biology.

Bacterial LPS consists of a polysaccharide region (itself consisting of an O-specific chain, an outer core and an inner core) covalently bound to a lipid A region which attaches LPS to the bacterial cell wall (reviewed by Malhotra and Bird, 1997). LPS may engage a number of specific receptors on monocytes and macrophages including CD14, β_2 leukocyte integrins, and Toll-like receptor 4 (reviewed by Ingalls et al., 1999). In the case of CD14 free LPS (represented here by grey ovals) is bound by a circulating protein called LPS-binding protein. This process initiates a conformational change increasing the binding capacity of LPS for CD14 (1). In contrast, free LPS has relatively low affinity for the receptor (2). Ligation of LPS with CD14 activates macrophages (3). Reference to Figure 2 shows that LPS can therefore effectively substitute for bacteria in models of neutrophilic pulmonary inflammation.

ligands on activated endothelial cells (Jutila et al., 1989); E-selectin production by endothelial cells is induced by inflammatory cytokines (Bevilacqua et al., 1987); and P-selectin is stored in endothelial cells and mobilised in response to endothelial stimulation (Bonfanti et al., 1989). It therefore appears that the degree of endothelial activation can be detected by circulating neutrophils, the selectins mediating "rolling" of neutrophils along endothelial surfaces in a way that permits further determination of the neutrophil's fate.

Under appropriate conditions, "rolling" neutrophils may be committed to firm adhesion as a consequence of the interaction between β_2 -integrins on neutrophils and ligands on activated endothelial cells, principal among which is ICAM-1 (Luscinskas et al., 1991). Three species of β_2 -integrin have been identified on neutrophils, namely CD11a/CD18, CD11b/CD18 and CD11c/CD18 (reviewed by Shanley et al., 1995). Of these, CD11b/CD18 surface expression can be up-regulated upon activation of neutrophils by signals such as C5a, leukotriene (LT)B₄, and fMLP (Hughes et al., 1992). Furthermore, neutrophil activation appears to induce conformational change in neutrophil β_2 -integrins, favouring firm adhesion to ICAM-1 (Kuijpers et al., 1992). The importance of interactions between β_2 -integrins and ICAM-1 is emphasised by the marked reduction in neutrophil migration upon blockade of these molecules (Arfors et al, 1987; Furie et al, 1991; Issekutz and Issekutz, 1992). The complexity and redundancy in inflammatory mechanisms can be appreciated however by the observation that CD11/CD18-independent neutrophil migration can occur (Issekutz et al., 1995).

The establishment of firm neutrophil-endothelial cell ligation results in migration of neutrophils through endothelial cell gap junctions in response to chemotactic gradients. A central role for early inflammatory cytokines in regulating neutrophil migration is invoked by the function of IL-1 in up-regulating both the expression of adhesion molecules and chemokines from endothelial cells (Luscinskas et al., 1991; Bittleman and Casale, 1995) The process of neutrophil extravasation in response to chemokines has received a great deal of attention, and is of specific relevance to work presented in Chapter 4. Before considering the mechanisms involved in greater detail however, it is worth considering that the neutrophil's journey from the endovascular compartment to the alveolus involves negotiation of not only endothelial gap junctions but extracellular matrix, alveolar epithelial cells, and epithelial lining fluid. Therefore, extrapolation of data from *in*

vitro studies assessing any one of these potential barriers should be interpreted with some caution.

Molecules with chemotactic activity for neutrophils (reviewed by Adams and Lloyd, 1997) can be derived from bacteria (eg fMLP), from complement (eg C5a) or from host cells (eg IL-8, growth-related oncogene α , epithelial-derived neutrophil attractant 78, granulocyte chemotactic protein-2, platelet activating factor (PAF), LTB₄). Interestingly, migration across epithelial barriers appears to occur preferentially in the basal-to-apical (ie physiological) direction (Parkos et al., 1991; Liu et al., 1996), and is dependent upon the nature of the chemotactic stimulus (Casale et al., 1992). Transepithelial migration also appears to be critically dependent on the origin of the epithelium, alveolar epithelium being more permissive than bronchial epithelium (Carolan and Casale, 1996). In keeping with the situation in endothelial cells, transepithelial migration appears to be largely (but not entirely) CD11b/CD18 dependent (Parkos et al., 1991). While the *binding* requirements for neutrophil migration are becoming clearer, considerable debate surrounds the issue of whether neutrophil *transmigration* is dependent upon cell-surface expression of proteolytic activity. This issue will be returned to in greater detail (particularly with respect to expression of HNE) in Chapter 4. In brief, the role of a number of neutrophil-derived enzymes has been examined in the context of migration across cellular barriers and matrix, but clear evidence supporting any single mechanism is currently lacking.

A further cautionary point in interpreting existing literature on neutrophil migration is that not all of the concepts described above may be applicable in the lung. For example, shear forces in the low pressure pulmonary arterial system differ from those in the systemic circulation, and pulmonary neutrophil extravasation has been shown to occur preferentially from capillaries rather than post-capillary venules (Downey et al., 1993). Furthermore, it should be noted that blockade of β_2 -integrins or depletion of L-selectin does not abolish pulmonary neutrophil migration (Mulligan et al., 1993a; Hogg and Doerschuk, 1995). Finally, it should be noted that the predominant cell type used in studies to elucidate mechanisms of neutrophil migration has been the human umbilical vein endothelial cell (HUVEC), with very little data pertaining to endothelial cells from the pulmonary vascular bed.

Once an extravasated neutrophil has reached the source of inflammation, it is required to locate and engulf pathogenic bacteria. This process of phagocytosis

commences as bacteria become bound to the neutrophil surface, when pseudopods extend around the bacteria effectively enclosing them within a "phagosome" which is itself enclosed within the neutrophil (reviewed by Murphy, 1976). Phagocytosis is considerably enhanced if the bacteria are opsonised with complement factors (eg C3b), or by IgG, the Fc portion of which is recognised by Fc receptors on the neutrophil surface (reviewed by Wright, 1992).

Critically, the specific and azurophil granules of the neutrophil rapidly fuse with internalised phagosomes, to produce membrane bound structures named phagolysosomes. Azurophil and specific granules each contain an impressive arsenal of bactericidal (and potentially cytotoxic) moieties (Table 1). In this way toxic antimicrobial products are brought into close proximity with bacteria, but remain isolated from the neutrophil's cytoplasm by means of a distinct membrane (Murphy, 1976). The means by which neutrophils destroy bacteria can be broadly divided into oxidative and non-oxidative mechanisms.

Oxidative mechanisms are characterised by the ability of stimulated neutrophils to increase oxygen uptake rapidly in tandem with activation of the NADPH oxidase enzyme (reviewed by Weiss, 1989). This enzyme catalyses the conversion of oxygen and NADPH to free oxygen radicals in the form of superoxide anion. Superoxide anion undergoes a dismutation reaction leading to the generation of hydrogen peroxide. In the presence of a second neutrophil granule enzyme, myeloperoxidase (MPO), hydrogen peroxide reacts with chloride ions to generate significant quantities of hypochlorous acid. Hypochlorous acid is a potent bactericidal agent. This sequence of reactions rapidly consumes NADPH, which in turn activates oxidation of glucose via the hexose monophosphate shunt (reviewed by Lehrer et al., 1988). As such the process of oxygen radical generation is energy intensive, and has been labelled the "respiratory burst".

In recent years, interest has increasingly been focused on non-oxidative mechanisms of bacterial killing. Several antimicrobial peptides reside in neutrophil granules (Table 1), some of which are discussed briefly here to illustrate the diversity of microbicidal activity of these cells (antimicrobial peptides are discussed in more detail in the relevant sections of Chapters 3, 5 and 6). Thus lysozyme is thought to exert antimicrobial activity by hydrolysis of a recurring linkage between N-acetylglucosamine and N-acetylmuramic acid residues in bacterial cell walls (reviewed by Lehrer et al., 1988), while lactoferrin is thought to effect antibacterial

	AZUROPHIL GRANULES	SPECIFIC GRANULES	OTHER GRANULES
ENZYMES	HNE Cathepsin G Proteinase 3 MPO N-acetyl- β -glucosaminidase Cathepsin B Cathepsin D β -glucuronidase β -glycerophosphatase α -mannosidase Peroxidase Acid phosphatase β -glucosaminidase Arylsulphatase Esterase	Collagenase Gelatinase Histaminase	Alkaline phosphatase Gelatinase N-acetyl- β -glucosaminidase Cathepsin B Cathepsin D β -glucuronidase β -glycerophosphatase α -mannosidase Acid phosphatase
ANTIMICROBIAL MOIETIES	Lysozyme Defensins BPI Azurocidin	Lysozyme Lactoferrin	

Table 1. Neutrophil granule contents.

A number of points should be considered when assessing these lists. Firstly, these are by no means exhaustive lists of neutrophil granule contents, and are designed to exemplify the range of potentially secreted toxins. Secondly, a significant proportion of the moieties listed have cytotoxic actions when released from neutrophils. Thirdly, a variety of other inflammatory mediators may be released from neutrophils, including PAF, leukotrienes, thromboxane and prostaglandins. Data is adapted from Baggiolini and Dewald (1985), Henson et al. (1992), and Bainton (1992).

activity principally by chelating the iron essential for bacterial viability (Oram and Reiter, 1968). Proteins found exclusively in azurophilic granules include bactericidal/permeability-increasing protein (BPI), a cationic peptide which disrupts membrane permeability in Gram-negative bacteria (Weiss et al., 1978), and cathepsin G which has activity against fungi as well as Gram-positive and Gram-negative bacteria (Lehrer et al., 1975; Shafer et al., 1986). However most attention in recent years has focused on the family of neutrophil defensins, small cationic peptides with an impressively broad spectrum of antimicrobial activity encompassing Gram-positive and Gram-negative bacteria, fungi and viruses (Ganz et al., 1985; reviewed by Lehrer et al., 1988). The study of the defensins has stimulated a hunt for further microbicidal peptides in neutrophils, and it seems likely that several more will be identified in the future.

Whilst considering the antimicrobial function of neutrophil peptides it is worth making brief mention of HNE, which is central to the work described in this thesis. Section 1.4.1. will describe HNE as a potent serine protease found in azurophil granules. In this setting it seems strange, at first glance, that a critical antimicrobial function for HNE was not identified early in the study of neutrophil biology. In fact, a body of evidence did gradually emerge to suggest an antimicrobial function for HNE, either as a direct effect (Thorne et al., 1976) or indirectly through the proteolytic activation of bactericidal molecules such as cathelicidins (Shi and Ganz, 1998; Cole et al., 2001). However perhaps the most persuasive argument for an antimicrobial function was provided by data describing an increased susceptibility of elastase "knock-out" mice to Gram-negative bacterial sepsis (Belaaouaj et al., 1998).

The efficiency with which neutrophils access target tissues and destroy bacteria is epitomised by acute Streptococcal pneumonia, in which affected alveoli are packed full with neutrophils and fibrinous inflammatory debris (see Figure 1). It is salient to recall that even before the advent of penicillin a significant proportion of patients with Streptococcal pneumonia survived without significant pulmonary sequelae, thus testifying to the neutrophil's incredible proficiency (for a description of the features of pneumonia in the pre-antibiotic era see Heath and Kay, 1980). The histological features of pneumonia do however serve as a reminder that neutrophils must be efficiently cleared from the site of inflammation without release of toxic contents. This is achieved through the process of apoptosis, a highly regulated sequence of events in which ageing neutrophils are recognised and phagocytosed by

resident macrophages without extracellular release of toxic neutrophil products (Savill et al., 1989).

1.3.3. Neutrophil-mediated lung injury, and the specific role of HNE.

Acute inflammation may resolve without residual tissue injury or dysfunction. However there is no doubt that acute inflammatory processes may alternatively damage tissues and lead to important pathological sequelae. This variability in outcome implies that acute inflammation may, under certain circumstances, be inappropriately or excessively stimulated and/or that endogenous defence mechanisms are deficient in the tissues of certain individuals. Against this background there are several reasons to implicate the neutrophil as a potential effector of tissue injury. Certainly neutrophils contain a myriad of potentially cytotoxic chemicals (Table 1), prominent tissue neutrophilia is associated with acute inflammatory tissue injury (reviewed by Malech and Gallin, 1987), and neutrophil influx is associated with inflammatory stimuli such as cigarette smoking (Hunninghake and Crystal, 1983). However the neutrophilia associated with acute tissue injury is not sufficient to establish a causative role for neutrophils, leading to the question of whether the neutrophil is specifically capable of effecting tissue injury? Certainly activated neutrophils may damage microvascular and pulmonary vascular endothelial cells (Smedley et al., 1986; Worthen et al., 1987; Westlin and Gimbrone, 1993), as well as pulmonary epithelial cells (Ayars et al., 1984). Furthermore, in established models of acute lung injury, neutrophil depletion has been shown to obviate tissue damage (Heflin and Brigham, 1981; Worthen et al., 1987).

Support for the concept that excessive neutrophil activation may be important in human lung injury is generally provided by reference to adult respiratory distress syndrome (ARDS) (reviewed by Windsor et al., 1983; Simon and Ward, 1992; Donnelly and Haslett, 1992). ARDS is characterised by an overwhelming and catastrophic neutrophil influx into alveoli, often in response to an extra-pulmonary stimulus such as pancreatitis, abdominal sepsis, or burns. The characteristic pulmonary neutrophilia is associated with significant degradation of the alveolar-capillary membrane, resulting in flooding of alveoli with proteinaceous exudate.

Further evidence for the role of neutrophils in acute inflammatory lung injury in humans is provided by the detection of active, cytotoxic neutrophil products in

airway secretions from patients with proven lung injury. It is highly likely that several of the toxins identified in Table 1 contribute to the pathophysiology of inflammatory lung conditions. However it is intriguing that one particular molecule, HNE, has been consistently implicated in the pathogenesis of lung disorders characterised by neutrophilia (reviewed by McElvaney and Crystal, 1997a). This has made HNE a potentially attractive target in attempts to ameliorate pulmonary injury. The following section (1.4.) will discuss HNE in detail, and will also briefly describe two further neutrophil serine proteases implicated in lung injury, namely proteinase 3 and cathepsin G.

Before doing so it is worth attempting to place the information provided so far in the appropriate context. Thus while the neutrophil plays an important part in acute inflammatory lung injury and HNE appears to be a central mediator of neutrophil-mediated damage, it must not be inferred that total ablation of neutrophils (or HNE) is a desirable therapeutic aim in inflammation. Indeed, the potentially devastating infective consequences of neutropenia are only too apparent clinically (Lekstrom-Himes and Gallin, 2000), and elastase deficiency is similarly associated with a propensity to Gram-negative sepsis (Belaouaj et al., 1998). The central paradox of acute inflammation - whether (and under which conditions) the neutrophil is beneficial or deleterious - therefore remains enormously relevant.

Furthermore, it must not be inferred that HNE is the single-most important mediator of inflammation. Figure 2 (itself simplified) testifies to the complexity and inherent redundancy in inflammatory mechanisms. It has therefore been argued that targeting of any single inflammatory mediator is unlikely to result in therapeutic benefit in inflammatory disorders. Nevertheless, it seems intuitively worthwhile to target important early mediators of inflammation (eg TNF- α or IL-1) and/or late effectors of damage such as HNE. Against this complex background it is important to realise that clinical trials attempting to neutralise IL-1 or TNF- α have had mixed outcomes, with generally favourable results in rheumatoid arthritis (reviewed by O'Dell, 1999; Yiang et al., 2000) and disappointingly poor results in sepsis (Fisher et al., 1996; Abraham et al., 1998; Vincent et al., 1999).

Finally, it is sobering to consider that severe inflammatory tissue injury may be effected in the relative absence of neutrophils and their products. For example acute lung injury is well described among neutropenic patients (Ognibene et al., 1986). Furthermore, although the neutrophil is the dominant cell type in sections and

lavage samples from patients with acute pulmonary injury, an important role for the alveolar macrophage has been suggested (Mass et al., 1972; Johnson and Ward, 1982; Sibille and Reynolds, 1990). While the experimental work in this thesis necessarily concentrates on the role of neutrophil products in effecting lung inflammation, it should be appreciated that alveolar macrophages may indeed contribute to lung injury, a point that will be stressed intermittently in later chapters.

1.4. SERINE PROTEASES DERIVED FROM NEUTROPHIL GRANULES

1.4.1. Neutrophil elastase.

The association between neutrophils and proteolytic activity appears to have been first described by Friedrich Müller (reviewed by Opie, 1922). However the enzyme now recognised as elastase was probably first partially characterised by Opie (1905), and named leucoprotease.

The single gene encoding HNE is a 4 kb gene on chromosome 19 (Zimmer et al., 1992). The 267 amino acid product is inactive but a small N-terminus fragment is cleaved by cathepsin C to yield the mature, proteolytically active protein (220 amino acids) (Sinha et al., 1987; McGuire et al., 1993). The molecule is glycosylated, has a molecular mass of 29 kDa, and is a basic molecule with a high arginine content (Bode et al., 1989). HNE is produced in undifferentiated myeloid precursors, and mRNA is not found in mature neutrophils (Fouret et al., 1989). HNE is stored in active form in azurophil granules, and it has been estimated that the concentration in each human neutrophil may approximate to 50 μ M (McElvaney and Crystal, 1997a).

HNE is a serine protease, the catalytic site being formed by three residues (histidine, aspartate and serine). The spatial arrangement of the catalytic site allows the serine residue to act as a nucleophile which can attack the carbonyl atom of selected peptide bonds, and particularly those formed by valine or alanine residues (Bode et al., 1989). The natural inhibitors of HNE are described in detail in the following section, but it is worth noting at this stage that four such inhibitors have been identified in the lung, in the form of α_1 -protease inhibitor (α_1 -PI), α_2 -macroglobulin (α_2 -M), secretory leukocyte protease inhibitor (SLPI) and elafin.

The binding characteristics of HNE predict for the molecule's wide substrate specificity (Table 2). With particular reference to tissue injury and inflammation, it is apparent that HNE can degrade components of extracellular matrix, several molecules of importance to acute and chronic inflammation, and some of its own natural inhibitors. As described in section 1.3.2. HNE is capable of destroying bacteria via direct (Belaouaj et al., 2000) and indirect mechanisms (Shi and Ganz, 1998), and it seems most likely that HNE evolved to perform an intracellular antimicrobial function.

The potential for extracellular HNE to damage lung tissue is suggested by the molecule's abundance in neutrophils, its broad substrate specificity, and the presence of active HNE in pulmonary secretions from patients with a variety of inflammatory disorders. Evidence for a role in mediating human disease was provided in classic papers describing a propensity for premature emphysema among patients deficient in α_1 -PI, the major circulating inhibitor of HNE (Laurell and Eriksson, 1963; Eriksson, 1964). The protease-antiprotease theory of emphysema arose from these observations. The theory contends that an excess of anti-elastases bathes the lung in health, but that during inflammation excessive or unopposed release of HNE may overwhelm prevailing levels of anti-elastase, leading to proteolytic destruction of tissue. Supportive evidence includes the significant negative correlation between total sputum elastase and the forced expiratory volume in one second (FEV₁) in chronic obstructive pulmonary disease (COPD) (Fujita et al., 1990; Vignola et al., 1998). The protease-antiprotease theory is controversial in some respects, and in particular because some patients with emphysema have normal anti-elastase activity in bronchoalveolar lavage fluid (BALF). Nonetheless the theory stimulated considerable interest in the association between HNE and pulmonary injury. Indeed active HNE has been described in airway secretions from patients with emphysema and chronic bronchitis (Fujita et al., 1990; Vignola et al., 1998), ARDS (Lee et al., 1981; Suter et al., 1992), cystic fibrosis (CF) (Goldstein and Döring, 1986; Suter et al., 1986; Birrer et al., 1994; Nunley et al., 1999), non-CF bronchiectasis (Stockley et al., 1984a), pneumonia (Boutten et al., 1996), bronchopulmonary dysplasia (Merritt et al., 1983), asthma (Vignola et al., 1998), and cryptogenic fibrosing alveolitis (Schaaf et al., 2000). Increased levels of HNE have also been described in serum from patients with established ARDS (Donnelly et al., 1995). Importantly, immunohistochemical studies have shown that HNE is detectable in the extracellular space in association with cleaved elastin in human bronchopneumonia (Watanabe et al., 1990).

Substrates cleaved by HNE

Elastin
Collagen types I-IV
Fibronectin
Laminin
Proteoglycans
Coagulation factors
Immunoglobulins
Complement proteins and receptors
Plasminogen
Pro-enzymes
CD14
IL-8
 α_1 -PI
SLPI

Cellular functions influenced by HNE

Increased secretion of mucus from serous cells
Impairment of ciliary function
Cytotoxicity against pulmonary epithelial cells

Table 2. HNE substrates, and the influence of HNE on respiratory cell function.
The lists described are not exhaustive. They are designed to illustrate the broad substrate specificity and cellular effects of HNE.

Supportive evidence for an effect of HNE in producing lung injury has also come from animal models. In particular, (intratracheal) IT instillation of HNE produces emphysema in dogs (Janoff et al., 1977), extracellular elastase has been shown to colocalise with alveolar structures in hamsters (Rudolphus et al., 1992), and neutrophil-mediated pulmonary injury has been prevented by administration of specific inhibitors of HNE (Rudolphus et al., 1993).

Against this background, inhibition of extracellular HNE seems a worthwhile therapeutic goal, and forms a major objective of the work described in this thesis.

1.4.2. Proteinase 3.

Proteinase 3 shares certain characteristics with HNE in that the genes for each molecule are situated close together on chromosome 19, both are neutral serine proteases, and both are produced in myeloid precursors then stored in an active form in azurophil granules (Kao et al., 1988; Rao et al., 1991; Sturrock et al., 1992). However while proteinase 3 is capable of degrading a variety of extracellular matrix proteins, its range of substrate specificity is less than that of HNE (Rao et al, 1991; reviewed by McElvaney and Crystal, 1997a). Furthermore, neutrophils are thought to contain less proteinase 3 than HNE (reviewed by Hiemstra et al., 1998).

Nonetheless, proteinase 3 is capable of producing emphysema when instilled IT in hamsters (Kao et al., 1988), and may potentially contribute to human disease. Two other notable features of proteinase 3 are the molecule's antimicrobial capacity (Gabay et al., 1989), and the specific association between autoantibodies to proteinase 3 and Wegener's granulomatosis (Ludemann et al., 1991).

1.4.3. Cathepsin G.

Cathepsin G is also a serine protease found in active form in azurophil granules and has approximately 35% homology with mature HNE (Barrett, 1981). In keeping with HNE, cathepsin G's range of substrate specificity is broad, targets including matrix proteins, immunoglobulin, fibrinogen and pro-enzymes contained in neutrophils (McElvaney and Crystal, 1997a). An antimicrobial effect has also been described for cathepsin G (Shafer et al., 1986). Although the concentration of cathepsin G in neutrophils closely resembles that of HNE, less is thought to be released upon neutrophil activation (Campbell et al., 1989).

1.5. THE INHIBITORS OF HNE

1.5.1. Overview.

The previous section outlined the evidence suggesting a prominent role for extracellular HNE in the generation of inflammatory lung injury. The available evidence suggests that endogenous anti-elastase defences may be overcome during inflammatory episodes, leaving HNE unopposed. A rational strategy in this context would be up-regulation and restoration of pulmonary anti-elastase activity during episodes of inflammation. In defining strategies aimed at selectively neutralising HNE in the lung, it is first necessary to understand the biology of endogenous pulmonary anti-elastases. In the following sections, the four existing anti-elastase molecules in the human lung will be discussed. Two of these (α_1 -PI and α_2 -M) are largely produced outwith the lung, and are thought to be relatively insensitive to conditions in the local pulmonary milieu. Greater attention will be paid to SLPI and elafin, low molecular weight inhibitors of HNE which are secreted by pulmonary epithelial cells in response to changes in the local pulmonary microenvironment. Particular emphasis will be placed on the biology of elafin. The interaction of elastase inhibitors with each other and regulation of their production by inflammatory cytokines will be considered. Finally, the application of synthetic elastase inhibitors to inflammatory models will be discussed.

1.5.2. Alpha₁-protease inhibitor.¹

As described previously, the association between pulmonary emphysema and serum deficiency of α_1 -PI led to recognition of the molecule's importance as a physiological inhibitor of HNE (Laurell and Eriksson, 1963; Eriksson, 1964; Gadek et al., 1981).

The human α_1 -PI gene is located on chromosome 14, and contains 5 exons and 4 introns (Long et al., 1984). The structure-function relationship of the gene has been reviewed by Sallenave et al. (1999a).

Alpha₁-PI is produced in hepatocytes (Perlino et al., 1987) and released into the circulation. It behaves as a classical acute phase protein, with serum levels increasing markedly in response to inflammatory stimuli (Aronsen et al., 1972). Up-

¹ Alpha₁-protease inhibitor is synonymous with alpha₁-antitrypsin.

regulation of secretion from hepatoma cells *in vitro* is induced by IL-6 (Perlmutter et al., 1989). The close association of α_1 -PI production with "secondary cytokines" mediating the systemic acute phase response may be of central importance to anti-elastase biology, as discussed in section 1.5.7. Alpha₁-PI is thought to diffuse into tissues from serum, and approximately 95% of α_1 -PI in the lung is thought to have a hepatic source (reviewed by McElvaney and Crystal, 1997b). Nevertheless, alternative cellular sources exist in the lung, including macrophages, neutrophils, and alveolar epithelial cells (Isaacson et al., 1979; Mornex et al., 1986; Venembre et al., 1994; Paakko et al., 1996; Sallenave et al., 1997a). Interestingly, induction of α_1 -PI secretion from alveolar epithelial cells is induced by oncostatin M but not by IL-6, IL-1 or TNF (Sallenave et al., 1997a).

Alpha₁-PI is secreted as a glycoprotein consisting of 394 amino acids, with a molecular mass of 52 kDa. Alpha₁-PI is a member of the serpin (serine protease inhibitor) family of inhibitors, and has the capacity to neutralise a wide range of serine proteases. However the principal function of α_1 -PI is to inhibit HNE. The molecules form a 1:1 stoichiometric complex, with an association rate constant of approximately $10^{-7}\text{M}^{-1}\text{sec}^{-1}$ (Beatty et al., 1980) and a dissociation constant of approximately 10^{-14}M (Loebermann et al., 1984). These observations suggest potent inhibition of HNE by α_1 -PI, leading to the conclusion that α_1 -PI is the most powerful inhibitor of HNE *in vivo* (McElvaney and Crystal, 1997b). The active site of α_1 -PI is situated at Met³⁵⁸-Ser³⁵⁹, and forms a bond with the catalytic site of HNE (Loebermann et al., 1984). The serpin-enzyme complex receptor identified on human mononuclear phagocytes may play an important part in clearance of α_1 -PI-HNE complexes (Perlmutter et al., 1990).

The biological properties of α_1 -PI have led to investigation of the molecule's potential therapeutic role in inflammatory conditions. Several such studies warrant attention. Intriguingly, α_1 -PI has been shown to protect mice against the lethal effects of TNF (Libert et al., 1996), suggesting an important role in the modulation of inflammation. In the specific context of human lung disease, aerosolised α_1 -PI has been shown to inhibit HNE and reconstitute the ability of BALF to neutralise pathogenic effects of *Pseudomonas aeruginosa* in CF (McElvaney et al., 1991). However the principal therapeutic focus has been on the hereditary deficiency of α_1 -PI (reviewed by Eriksson, 1996).

Consideration of the severe (PiZZ) hereditary form of α_1 -PI deficiency and its treatment is instructive in the context of this thesis for a number of reasons. Firstly it is clear that a significant proportion of individuals with PiZZ deficiency do not develop emphysema, based on evidence from large Scandinavian and North American registries (Eriksson, 1996; The Alpha- $_1$ -Antitrypsin Deficiency Registry Study Group, 1998). One interpretation of these data is that, at least in a sub-population of patients, alternative elastase inhibitors may be sufficient to prevent clinical lung disease. Secondly, although evidence exists to suggest that augmentation therapy using recombinant α_1 -PI slows decline of FEV $_1$ (The Alpha- $_1$ -Antitrypsin Deficiency Registry Study Group, 1998), the only randomised, double-blind placebo controlled trial reported to date did not show a statistically significant improvement with replacement therapy (Dirksen et al., 1999). This may have implications for the optimal mode and route of delivery of α_1 -PI, and/or for the effectiveness of α_1 -PI in preventing lung injury mediated by HNE. Finally, as a single gene defect, α_1 -PI deficiency is potentially amenable to correction with somatic gene therapy. The principles of gene therapy are discussed in detail in section 1.6., but it is worth mentioning that α_1 -PI gene transfer to the airways is eminently feasible (Rosenfeld et al., 1991; Canonico et al., 1994), and that trials of α_1 -PI gene therapy are under way.

The absence of unequivocal evidence to support a protective effect of α_1 -PI replacement therapy raises questions as to whether the desirable inhibitory effects of α_1 -PI *in vitro* are reproduced *in vivo*. Indeed optimal inhibitory characteristics may not prevail under conditions of acute inflammatory stress for at least three important reasons. Firstly, the methionine in the active site of α_1 -PI is susceptible to oxidation and a marked reduction in activity can be induced by biologically relevant oxidants (Vogelmeier et al., 1997). Secondly, it appears that α_1 -PI may be extensively cleaved in inflammatory conditions such as CF and chronic bronchitis (McElvaney and Crystal, 1997b). Thirdly, evidence suggests that α_1 -PI may be unable to access the gap between neutrophil surface and HNE substrate (Campbell and Campbell, 1988; Owen et al., 1995), which may take on considerable importance on the basis that HNE can be expressed at the neutrophil surface membrane (Owen et al., 1995) and that neutrophil adhesion is a powerful stimulus for granule exocytosis (Dahinden et al., 1983).

Extensive efforts have been made to overcome potential limitations of α_1 -PI for therapeutic use, including the engineering of oxidation-resistant mutants. On the

basis of available evidence it may be reasonable to conclude that α_1 -PI retains considerable potential as a therapeutic tool in pulmonary inflammation (especially when limitations relating to mode of delivery and biochemical structure are resolved). Against this background however, examination of the function of alternative anti-elastases seems particularly important.

1.5.3. Alpha₂-macroglobulin.

Alpha₂-M is a very large glycoprotein (molecular mass 720 kDa) with the capacity to inhibit a wide variety of proteases, including HNE (reviewed by McElvaney and Crystal, 1997b). Alpha₂-M is produced in hepatocytes (Munck Petersen et al., 1988) and secreted into the circulation from where it is thought to diffuse into tissues. However the considerable size of α_2 -M limits its concentration in the lung to molar concentrations below 1% of those described for α_1 -PI under basal conditions (McElvaney and Crystal, 1997b). By virtue of its low concentration in lung, α_2 -M seems unlikely to have a significant role in the immediate inhibition of extracellular HNE. In this setting it is interesting that alternative functions have been suggested for α_2 -M, including a role in enhancing antigen presentation (Chu et al., 1994).

1.5.4. Secretory Leukocyte Protease Inhibitor.²

The observation that α_1 -PI could not account for all of the anti-elastase activity of sputum ultimately led to the description and partial characterisation of a low molecular weight, acid stable "bronchial mucus inhibitor" (synonymous with SLPI) capable of reducing the proteolytic activity of neutrophil extracts (Hochstrasser et al, 1972). The molecule was shown to be an inhibitor of HNE (Ohlsson and Tegner, 1976). SLPI was subsequently shown to be widely distributed in human tissues, being detectable in bronchial, nasal, salivary, tear, cervical and seminal secretions (reviewed by McElvaney and Crystal, 1997b; Sallenave et al., 1999a).

The human SLPI gene spans approximately 2.65 kb, comprising 4 exons and 3 introns (Seemuller et al., 1986; Thompson and Ohlsson, 1986). A promoter region

² SLPI has also been known as antileukoprotease, mucus proteinase inhibitor, and bronchial mucus inhibitor.

has been identified (Maruyama et al., 1994), within which resides a short sequence conferring lung specific expression (Kikuchi et al., 1997).

SLPI is a 107 amino acid cationic protein, which is non-glycosylated, and has a molecular mass of 11.7 kDa (Seemuller et al., 1986; Thompson and Ohlsson, 1986). The molecule is arranged in a compact, "boomerang" shape, and is characterised by the presence of two structural domains each containing four disulphide bridges (Grütter et al., 1988). The NH₂-terminal domain contains heparin-binding sites (Ying et al., 1994; Mellet et al., 1995), while the active site resides in the COOH-terminal domain (Grütter et al., 1988). The reactive site loop contains the scissile peptide bond Leu⁷²-Met⁷³ (Grütter et al., 1988). SLPI is capable of inhibiting HNE, cathepsin G, trypsin, chymotrypsin and mast cell chymase, but does not inhibit proteinase 3 (reviewed by Sallenave et al., 1999a). SLPI binds HNE tightly with 1:1 stoichiometry (Boudier and Bieth, 1989 and 1992). Importantly the size and compact structure of SLPI may contribute to its ability to access the gap between neutrophil and substrate (Rice and Weiss, 1990), and human SLPI has been shown to associate with elastic fibres in pulmonary connective tissue (Willems et al., 1986). However, as in the case of α_1 -PI, the methionine in the active site is susceptible to oxidation with associated reduction of activity (Vogelmeier et al., 1997).

Importantly the regulation of expression of SLPI appears to differ from that of α_1 -PI in that IL-1 and TNF- α effect significant up-regulation of secretion from alveolar epithelial cells (Sallenave et al., 1994; 1997a). This suggests a differential response of elastase inhibitors to cytokines, a theme which is returned to in section 1.5.7. Intriguingly, HNE and cathepsin G stimulate expression of SLPI mRNA in pulmonary epithelial cells (Abbinante-Nissen et al., 1993; Sallenave et al., 1994), but reduce secretion of SLPI protein (Sallenave et al., 1994). SLPI expression may also be up-regulated by corticosteroids (Abbinante-Nissen et al., 1995).

The main physiological function of SLPI is thought to be inhibition of HNE, and the higher concentrations of SLPI associated with upper airway secretions have suggested an important role in protection of the proximal respiratory tract (Sallenave et al., 1994; reviewed by McElvaney and Crystal, 1997b). These features prompted studies testing the protective potential of SLPI in models of inflammation. IT recombinant human SLPI was found to ameliorate alveolitis induced by immune complexes in rats (Mulligan et al., 1993b) and emphysema induced by LPS in hamsters (Rudolphus et al., 1993). A truncated form of recombinant human SLPI

reduced lung damage mediated by HNE or by sequential administration of LPS and fMLP in hamsters, the effect being maintained whether the SLPI was administered IT or intravenously (IV) (Mitsuhashi et al., 1997). Furthermore, intraperitoneal (IP) truncated recombinant human SLPI significantly inhibited the development of bleomycin-induced pulmonary fibrosis in hamsters (Mitsuhashi et al., 1996).

Recombinant human SLPI was found to be safe when administered to patients with CF, and a seven day course was associated with a reduction in airway neutrophilia, HNE, and IL-8 concentration (McElvaney et al., 1992).

These very encouraging early results suggested therapeutic applications for SLPI. Interestingly, these data were soon to be complemented by several strands of evidence suggesting alternative anti-inflammatory functions for SLPI.

In particular the model of immune complex alveolitis developed by Ward and colleagues (Mulligan et al., 1993b) has been instructive in understanding SLPI biology. Thus recombinant human SLPI increases I κ B β (an endogenous inhibitor of the pro-inflammatory nuclear factor NF κ B) in the rat lung (Lentsch et al., 1999), the effect apparently being dependent on the capacity of SLPI to inhibit trypsin (Mulligan et al., 2000). Furthermore endogenous rat SLPI appears to protect against lung injury by reducing the generation of C5a (Gipson et al., 1999).

Considerable attention has similarly focused on the anti-inflammatory role of SLPI in monocyte/macrophage biology. SLPI has been shown to inhibit the signalling pathways leading to generation of matrix metalloproteinases in LPS-stimulated human monocytes, the effect being independent of antiprotease activity (Zhang et al., 1997). Similarly Ding and colleagues propose that SLPI serves to reduce the pro-inflammatory response of macrophages to LPS. Their data suggest that murine macrophage SLPI is induced by LPS (Jin et al., 1998) and that murine SLPI is constitutively overexpressed in an LPS-hyporesponsive murine macrophage cell line (Jin et al., 1997). In this context it is interesting that SLPI transfected macrophages can interfere with the uptake of LPS (Ding et al., 1999). Furthermore LPS-responsive macrophages can be rendered hyporesponsive by prior SLPI transfection (Jin et al., 1997). Interestingly, LPS hyporesponsive monocytes from patients with sepsis can be reactivated by interferon- γ . Against this background complex mechanisms regulating SLPI production are suggested by the observation

that the pro-inflammatory transcription factor interferon regulatory factor-1 reduces SLPI expression (Nguyen et al., 1999).

Extending the anti-inflammatory role of SLPI still further, recombinant human SLPI has been shown to inhibit both elastase- and allergen-induced airway hyperresponsiveness (Suzuki et al., 1996; Fath et al., 1998; Wright et al., 1999).

Finally, the plethora of novel anti-inflammatory functions ascribed to SLPI can be extended both to a direct antimicrobial function (discussed in detail in Chapter 5), and to a profound effect on normal wound healing. The latter effect was identified in SLPI "knock-out" mice in which skin wounds healed slowly with impaired matrix accumulation, and persistence of inflammatory cells (Ashcroft et al., 2000).

The multiple anti-inflammatory functions identified for SLPI clearly suggest a variety of novel therapeutic strategies. However it is worth noting that therapeutic implementation of SLPI may not be devoid of problems. In particular recombinant full-length human SLPI increases the absolute partial thromboplastin time (APTT) in animal models (Masuda et al., 1995), raising the possibility that systemic administration of SLPI may predispose to haemorrhage. In addition, the data outlined above raise theoretical concerns that significant over-expression of SLPI could lead to immune suppression.

1.5.5. Elafin.³

The early understanding of elafin biology resulted, to a large extent, from the convergence of data derived from studies in skin and in bronchial secretions. The specific dermatological implications for elafin will be discussed only briefly in this section, and greater detail can be extracted from the works of Schalkwijk, Molhuizen, Wiedow, and Christophers, (early data is reviewed by Molhuizen and Schalkwijk, 1995).

From a pulmonary perspective, the characterisation of SLPI was greeted with great interest, as discussed above. However it remained clear that α_1 -PI, α_2 -M and SLPI could not account for all of the anti-elastase activity in airway secretions from

³ Elafin has also been known as elastase-specific inhibitor, skin-derived antileukoprotease, and trappin-2.

healthy individuals (Boudier et al., 1987) and patients with chronic bronchitis (Morrison et al., 1987). The existence in bronchial secretions of a further low molecular weight antiprotease capable of inhibiting HNE and porcine pancreatic elastase (PPE) but not cathepsin G had already been described by Hochstrasser et al. (1981) and by Kramps and Klasen (1985). Against this background the inhibitor was isolated and purified from the sputum of patients with chronic bronchitis (Sallenave and Ryle, 1991). The molecule inhibited HNE rapidly and strongly, showed considerable homology with SLPI, and the putative active site was identified as an Ala-Met bond (Sallenave and Ryle, 1991). At around the same time Wiedow et al. (1990) described a low molecular weight inhibitor of HNE and PPE isolated from psoriatic skin (which they called elafin), whilst Schalkwijk and colleagues isolated such an inhibitor from psoriatic scales and human keratinocytes (Schalkwijk et al., 1990; Schalkwijk et al., 1991).

The moiety isolated from sputum by Sallenave and Ryle (1991) was found to cross-react immunologically with the species described by Kramps and Klasen (1985), and to have almost complete sequence homology with Wiedow's molecule (Sallenave et al., 1992). The gene encoding elafin was cloned and sequenced by Sallenave and Silva (1993) and codes for a 117 amino acid protein, the first 22 amino acids of which represent signal peptide (Sallenave and Silva, 1993). There is now little doubt that elafin can be proteolytically cleaved into smaller fragments, that the various fragments described in the foregoing studies were derived from the same molecule, and that the species described by Hochstrasser et al. (1981) and by Kramps and Klasen (1985) was indeed elafin.

It is now clear that elafin is a potent inhibitor of the elastolytic enzymes HNE, PPE and proteinase 3 (Wiedow et al., 1990; Sallenave and Ryle, 1991; Wiedow et al., 1991; Ying and Simon, 2001), but does not inhibit cathepsin G, trypsin, chymotrypsin or plasmin (Wiedow et al., 1990; Tsunemi et al., 1992). As such there are important differences in the inhibitory profiles of elafin, SLPI and α_1 -PI (reviewed by McElvaney and Crystal, 1997b). Elafin secretion in the human lung may be associated with production by alveolar epithelium, Clara cells, or tracheal epithelium (Sallenave et al., 1993; Nara et al., 1994). Interestingly, studies of the tissue localisation of elafin have found that expression is largely confined to epithelial surfaces (Nara et al., 1994; Pfundt et al., 1996), although low levels of expression have been described in neutrophils (Sallenave et al., 1997b) and vascular smooth muscle (Nara et al., 1994).

The established structure of elafin has considerable implications for function. Elafin is a cationic and relatively acid-stable protein which is not glycosylated and has approximately 40% sequence homology with SLPI (Wiedow et al., 1990; Sallenave and Silva, 1993). The molecule has two distinct structural domains. The COOH-terminal domain is compact and robust on account of four disulphide bonds, and contains the Ala-Met scissile bond forming the active inhibitory site (Tsunemi et al., 1996). Elafin and HNE form a fully reversible 1:1 stoichiometric complex (Ying and Simon, 1993) but the bond formed is tight with a relatively slow rate of dissociation (Sallenave and Ryle, 1991; Ying and Simon, 1993; Tsunemi et al., 1996) and elafin may therefore best be regarded as a "pseudo-irreversible inhibitor". Elafin also forms a fully reversible 1:1 stoichiometric complex with proteinase 3, though elafin appears to dissociate more rapidly from proteinase 3 than from HNE (Ying and Simon, 1993, 2001). The methionine in the active site of elafin predicts for potential oxidation with loss of function. This effect has not been confirmed to date, though elafin has been shown to be inactivated by PPE and sputum from patients with CF (Sallenave et al., 1995).

The NH₂-terminal domain is characterised by repeated VKGQ motifs (Sallenave and Silva, 1993). This sequence acts as a substrate for transglutaminase with the resultant formation of covalent isopeptide bonds between glutamine and lysine. Transglutaminase has been shown to effect polymerisation of elafin and the covalent binding of elafin to laminin *in vitro* (Nara et al., 1994), raising the intriguing possibility that tissue transglutaminase may anchor elafin to extracellular matrix *in vivo*. In this context it may be important that elafin has been identified in cross linkage with loricrin in the cornified cell envelope of epithelial cells (Steinert and Marekov, 1995),

Interestingly the four-disulphide core and transglutamination sites found in elafin are structural determinants of homologous proteins including sodium-potassium ATPase, and recently identified porcine proteins. This led to the concept that these molecules belong to a structural family, recently named the "trappin family" (reviewed by Schalkwijk et al., 1999). This association may suggest additional functions for elafin, but it remains pertinent to recall the similarities between elafin and SLPI (which is not a trappin) which are both secreted, cationic inhibitors of HNE with considerable sequence homology, a similar reactive site (Ala-Met and Leu-Met respectively), a four-disulphide COOH-terminal domain, and binding sequences in the NH₂-terminal domain.

Elafin is encoded by a single gene located on chromosome 20 (Molhuizen et al., 1994) which spans approximately 2.3 kb, and contains 3 exons and 2 introns (Sallenave and Silva, 1993). A number of putative transcription factor binding sites have been identified 5' of the elafin gene, and reporter gene constructs have suggested the presence of positive and negative regulatory sequences for elafin expression in mammary epithelial cells (Zhang et al., 1995). However the genetic sequences regulating elafin expression remain poorly understood and require further characterisation.

Importantly IL-1 and TNF- α are capable of up-regulating expression and secretion of elafin from pulmonary epithelial cells (Sallenave et al., 1994), the response being mediated largely by interaction between NF κ B and a proximal promoter region (Bingle et al., 2001). The up-regulation is particularly striking after the application of IL-1 to alveolar epithelial cells (Sallenave et al., 1994). These findings may imply a central role for elafin in airway protection during the earliest phase of inflammatory processes, a point which is returned to in section 1.5.7. Interestingly HNE up-regulates expression of elafin mRNA in A549 cells, but secretion of elafin is in fact down-regulated by the application of HNE (Reid et al., 1999). While pro-inflammatory mediators generally appear to up-regulate expression of the elafin gene, elafin expression in psoriatic skin may be down-regulated by the application of topical anti-inflammatory corticosteroids (Kuijpers et al., 1997).

The relative importance of elafin in the clinical setting has been investigated by obtaining airway secretions from patients with a variety of pulmonary inflammatory disorders. These studies have suggested that elafin constitutes approximately 20% of the total anti-elastase antigen retrieved from BALF during health (Tremblay et al., 1996). No specific hereditary deficiency of elafin (or SLPI) has yet been described, but it is interesting that the relative contribution of elafin to the total concentration of elastase inhibitors is reduced in airway secretions from patients with CF, COPD, asthma, and ARDS associated with sepsis (Sallenave et al., 1999a and 1999b). In all of these conditions SLPI appears to be disproportionately up-regulated. The relative reduction in elafin is particularly striking in CF and COPD (Sallenave et al., 1999a). In contrast elafin levels are increased in BALF from patients with farmers lung, a form of extrinsic allergic alveolitis characterised by BALF lymphocytosis (Tremblay et al., 1996). It is certainly intriguing that the relative contribution of elafin should be reduced in conditions associated with airway

neutrophilia and airway elastase activity. However the clinical studies should be interpreted cautiously given the small patient numbers, heterogeneity of patients, and lack of activity data for individual inhibitors. The situation becomes still more complex in light of reports suggesting that a proportion of SLPI and elafin in BALF may be cell associated (Griese et al., 1997).

In order to characterise the role of elafin in inflammation (and with a view to exploiting properties of elafin therapeutically), several groups have applied elafin in animal models of inflammatory injury. These findings are discussed in detail in Chapters 4 and 6 but in brief, studies thus far have concentrated on the effects of elafin in models of vascular, myocardial, and skeletal muscle injury, an excess of exogenous (human) elafin generally resulting in tissue protection (Crinnion et al., 1994; Cowan et al., 1996; Tiefenbacher et al., 1997; Zaidi et al., 1999; O'Blenes et al., 2000; Barolet et al., 2001).

1.5.6. Other inhibitors of HNE.

The four principal inhibitors of HNE identified in human airway secretions are discussed above. Potential therapeutic applications are implied by the properties of these inhibitors, particularly in the context of HNE-mediated disease. When considering the therapeutic merits of pulmonary inhibitors of HNE it is worth noting however that other natural and synthetic inhibitors of HNE exist. Details relating to the biochemistry and *in vivo* effects of the many important elastase inhibitors which are not native to the lung will not be discussed in the forthcoming sections of this work (these can be found in reviews by Powers, 1983; Schnebli and Braun, 1986; Snider et al., 1994). However it should be recorded that three particular groups of inhibitors have played an important part in characterising the biology of HNE, namely eglin C (a natural inhibitor derived from leeches), chloromethyl ketones, and a compound named ONO-5046, which has been used to characterise the contribution of elastase to several animal models of lung disease including pulmonary fibrosis (Taooka et al., 1997) and bronchial hyperreactivity (Fujimoto et al., 1995).

1.5.7. The regulation of pulmonary elastase inhibitors during inflammation.

The mechanisms regulating each of the important pulmonary elastase inhibitors suggest that inhibition of HNE may be co-ordinated by the inflammatory process (reviewed by Sallenave et al., 1999a and outlined schematically in Figure 4).

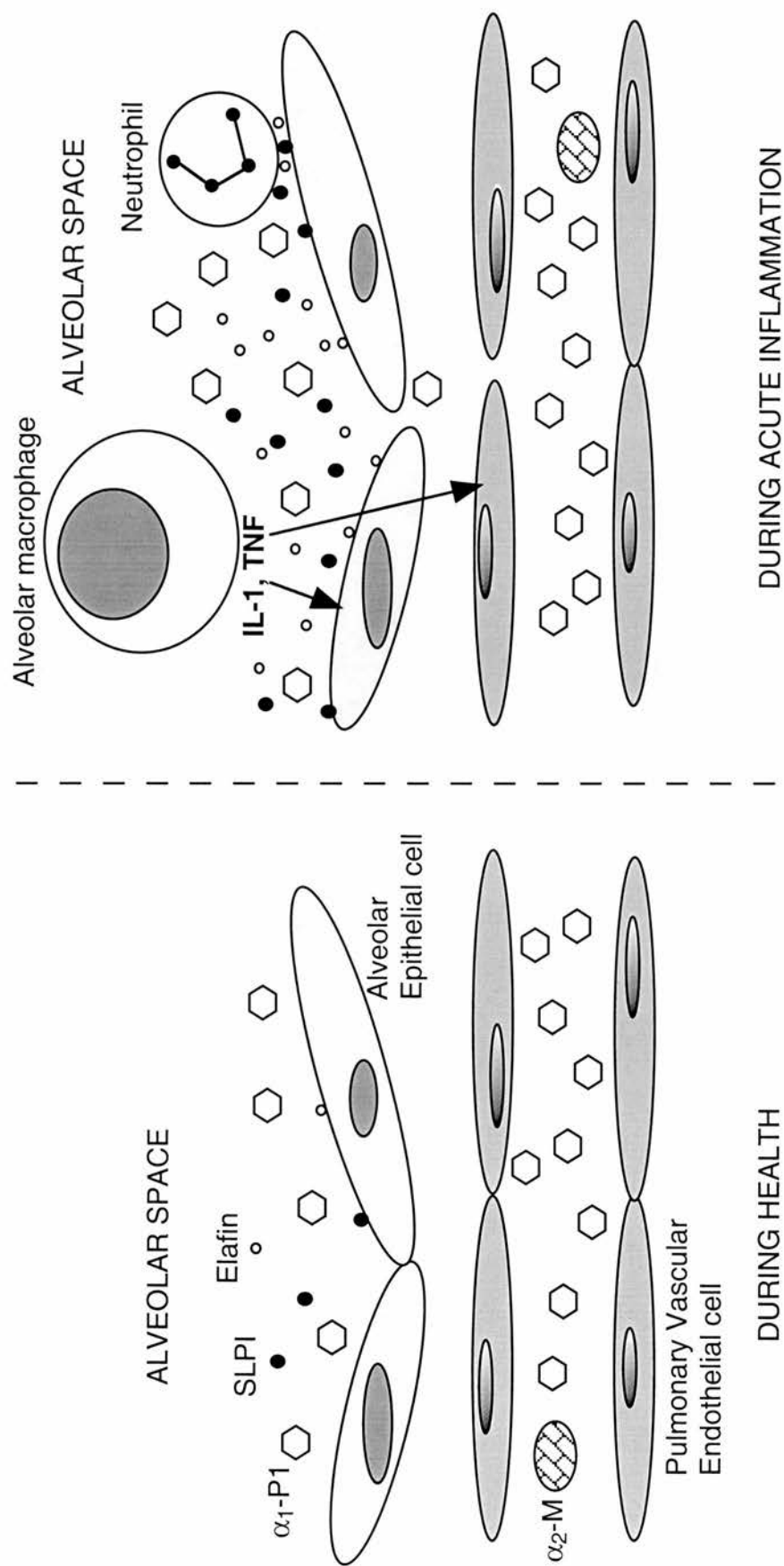


Figure 4. Regulation of pulmonary anti-elastases.

During health (left hand panel) α_1 -PI (hexagons) diffuses from the circulation to the alveolar space. On account of its size, much less of the larger α_2 -M (lattice ovoid) diffuses into alveoli. Relatively small concentrations of the low molecular weight inhibitors SLPI (black dots) and elafin (open circles) are secreted, and α_1 -PI is the predominant moiety in alveoli. However, unlike α_1 -PI, SLPI and elafin may have the capacity to bind host tissue. During early inflammatory responses (right hand panel), IL-1 and TNF stimulate endothelial and epithelial cells, and rapidly up-regulate expression of elafin and SLPI from alveolar epithelium. Concentrations of α_1 -PI increase in view of the leaky alveolar-capillary membrane, but production of α_1 -PI is not up-regulated until stimulated by "secondary" cytokines such as IL-6. Furthermore, elafin and SLPI (but not α_1 -PI) may be able to access the neutrophil-substrate interface.

It seems plausible that elafin and SLPI are up-regulated very early in the inflammatory response, on the basis that they are produced in local epithelium, and their secretion is rapidly initiated by the early inflammatory cytokines IL-1 and TNF- α (Sallenave et al., 1994). The induction of "secondary" cytokines such as IL-6 may then up-regulate α_1 -PI secretion from the liver (Perlmutter et al., 1989).

Interestingly, it has been demonstrated that HNE bound to SLPI can be transferred to α_1 -PI (Fryksmark et al., 1983), and that the α_1 -PI-HNE complex can be cleared by macrophages (Perlmutter et al., 1990). The rapid up-regulation, tissue-binding capacity and size of elafin and SLPI may therefore suggest a role for these molecules in neutralising HNE generated adjacent to cellular and interstitial substrates, while α_1 -PI may exist to scavenge remaining HNE and to effect the ultimate clearance of HNE from the lung. This process requires further clarification, but in the meantime provides an intriguing model.

1.5.8. The attractions of elafin augmentation as a strategy for the amelioration of inflammatory lung injury.

The information provided earlier in this chapter suggested that inhibition of extracellular HNE is a desirable therapeutic goal in pulmonary inflammation. The discussion of elastase inhibitors leads to the important question of which to augment in order to prevent inflammatory lung injury?

For several reasons, elafin was felt to be a particularly good candidate for the purposes of this work. Potential advantages over α_1 -PI include the local production of elafin and the lower molecular mass. Potential advantages over SLPI include the observation that elafin is particularly effective against the "elastolytic" neutrophil enzymes (SLPI does not inhibit proteinase 3), and theoretical concerns relating to immunosuppressive effects and coagulopathy with the use of SLPI. Additionally, it has been suggested that SLPI functions primarily to protect proximal airways; in the context of alveolar epithelial cells it is interesting that, relative to baseline values, IL-1 up-regulates elafin far more than SLPI (Sallenave et al., 1994), potentially suggesting a greater importance for elafin in protecting distal airways. Finally, the profound relative deficiency of elafin in conditions such as COPD may suggest an argument for its repletion in such disorders.

The remaining question is how best to deliver elafin to the airway? For reasons discussed in the following section, gene therapy seems ideally suited for this purpose.

1.6. PRINCIPLES OF GENE THERAPY

1.6.1. Overview and comparison with delivery of recombinant protein.

Gene therapy may be defined as the transfer of genetic material to effect a desired biological response. In practical terms the genetic material is usually a therapeutic transgene engineered to be under the control of a selected promoter region of DNA, the entire genetic material being enclosed in a "vector" with the inherent capacity to access target cells and deliver its genetic contents. The last fifteen years has seen a huge proliferation of interest in gene therapy, with respiratory applications occupying a prominent position in the field on account of the ready accessibility of the airways, and the fact that CF is the commonest single gene defect among Caucasians (reviewed by Greening, 2000). Although pulmonary gene therapy expanded largely in response to the desire to correct the single gene defect of CF, gene therapy is being applied to increasingly complex respiratory disorders, as discussed later in this chapter (reviewed by Curiel et al., 1996; Alton and Geddes, 1997; Simpson et al., 2000).

A central question in considering gene therapy strategies for lung disease is whether gene therapy offers advantages over more traditional modes of augmentation such as administration of recombinant protein. In the context of augmenting elafin levels in and around those pulmonary cells at risk of HNE-mediated damage, gene therapy has important advantages over recombinant protein. Firstly, recombinant proteins generally have a short half-life, usually measured in a few hours (Stolk et al., 1995) thus requiring regular administration, with attendant implications for cost. In contrast gene therapy can potentially produce therapeutic concentrations for many months (Snyder et al., 1997). Secondly, it is hard to target specific cells or to control levels of protein in tissues using recombinant protein. Infusion of recombinant protein often leads to peaks of protein concentration in tissues shortly after administration and consequent risk of adverse effects. In contrast gene therapy offers the potential to use promoters which may be inducible (Gossen et al., 1995; No et al., 1996) and/or tissue- or cell-specific (Strayer et al., 1998).

Interestingly, in the context of anti-protease biology, α_1 -PI gene therapy has recently been shown to be potentially more efficacious than recombinant protein in α_1 -PI deficient patients (Brigham et al., 2000).

Gene therapy strategies have traditionally been classified as "viral" or "non-viral" according to the vector system used (Table 3). With reference to pulmonary disease, the predominant gene therapy vectors have been cationic liposomes and recombinant adenoviruses. The following sections will outline the vectors available for gene transfer with discussion of their advantages and disadvantages. Particular emphasis will be placed on the pulmonary application of recombinant adenoviruses. The clinical application of pulmonary gene therapy will be discussed, as exemplified by trials in patients with CF.

1.6.2. Non-viral gene therapy.

Cationic liposomes are the most extensively studied vectors in non-viral gene therapy. Their use rests on the principle that anionic DNA (or RNA) may be bound within cationic liposomes. Liposome-DNA complexes usually consist of a charged head group (which interacts electrostatically with nucleic acid), a linker, and a hydrophobic domain which determines packaging of the complex (Felgner et al., 1987; Smith et al., 1993). The liposome is incorporated into negatively charged cell surfaces, with subsequent release of genetic material into the cytoplasm (reviewed by Schofield and Caskey, 1995; Curiel et al., 1996). The advantages of cationic liposomes in gene therapy protocols are ease of production, the almost unlimited capacity to carry DNA, and the relative lack of immunogenicity allowing repeat administration (Table 3). The major disadvantage is the low rate of transfection and gene expression achieved using liposomes. This is thought to reflect destruction of DNA in endosomes (Crystal, 1995). Furthermore, high doses of cationic liposomes have been shown to provoke significant inflammation (Scheule et al., 1997). As described in section 1.8. cationic liposomes have been applied with some degree of success in clinical trials (Porteous et al., 1997; Alton et al., 1999). However at the time the experimental work in this thesis commenced, the inefficiency of transfection associated with cationic liposomes was felt to outweigh the potential advantages of these vectors.

Before considering viral vectors however, it should be noted that non-viral gene therapy strategies are evolving rapidly, and offer potential future applications in

A.

VIRAL VECTORS	NON-VIRAL VECTORS
ADENOVIRUS* ADENO-ASSOCIATED VIRUS RETROVIRUSES LENTIVIRUSES SENDAI VIRUS	CATIONIC LIPOSOMES* LIGAND-DNA CONJUGATES

B.

	ADENOVIRUS	CATIONIC LIPOSOME
Capacity to produce high yield in the lab	High	High
DNA carrying capacity	E1-, partially E3-deleted virus ~ 8 kb "Gutted" virus ~ 30 kb	Practically unlimited
Transfection efficiency in the lung	High	Low
Duration of expression in the lung	Transient	Transient
Host inflammatory response in the lung	Intense, limiting re-administration	Mild; re-administration feasible

Table 3. Vectors used in pulmonary gene therapy.

A. Vectors applied in clinical and/or laboratory pulmonary gene therapy protocols.

* denotes the most commonly applied vectors in respiratory gene therapy.

B. A comparison of the relative advantages and disadvantages of adenoviral and liposomal vectors. The high natural tropism of adenovirus for respiratory epithelium makes this vector extremely attractive, particularly for protocols requiring transient expression of transgene.

the lung and other organs. Interestingly, simple "naked" DNA (ie not enclosed in a vector) may lead to striking gene expression under certain circumstances, exemplified by the improvement in ischaemic ulcers after single intramuscular injection of plasmid DNA encoding the vascular endothelial growth factor (VEGF) gene (Baumgartner et al., 1998). Similarly conjugation of DNA with ligands identifying specific cell surface receptors may allow enhanced gene delivery to cells in the lung (Curiel et al., 1992; Curiel et al., 1996; Jenkins et al., 2000).

Finally, the traditional boundaries between non-viral and viral gene delivery have been crossed in recent years by strategies aiming to capitalise on the advantages of each. Thus receptor-mediated gene transfer may be enhanced by incorporation of viral structures involved in nuclear localisation (Cristiano et al., 1993), and the efficiency of viral transfection may be combined with the high DNA carrying capacity of liposomes in Sendai virus-liposome complexes (Schofield and Caskey, 1995; O'Blennes et al., 2000).

1.6.3. Viral gene transfer.

Adenoviruses are ideally suited to the transfer of transgenes to the respiratory tract on account of their natural tropism for respiratory epithelium and their ability to transfect non-dividing airway epithelial cells (reviewed by Kremer and Perricaudet, 1995). A separate section (1.7.) is therefore devoted to adenoviral gene therapy, while this section will briefly consider other viruses of importance to gene therapy protocols.

In this respect, retroviruses occupy an important position, and were used in the significant majority of early clinical gene therapy trials. Advantages of retroviruses include the ability to integrate therapeutic transgene into the host genome thus establishing stable transfection (Miller, 1992). However retroviruses largely depend upon active cell division to achieve infection (Miller, 1992). Whilst these properties make retroviruses attractive for targeting tumour cells, the relatively terminal differentiation of cells lining the respiratory tract and pleural cavity has largely precluded the use of retroviruses in respiratory applications other than cancer (Roth et al., 1996; Tan et al., 1996).

In contrast the lentivirus genus of retroviruses (which include human immunodeficiency virus 1 (HIV-1)) may find a more prominent place in respiratory

gene therapy on account of the ability of lentiviruses to transfect non-dividing cells (Naldini et al., 1996; Goldman et al., 1997a; Buchschacher and Wong-Staal, 2000). However concerns remain over the consequences of integrating genetic material into the host genome (Poeschla et al., 1998).

Finally, AAV vectors show signs of making a significant impact in respiratory gene therapy. AAV is a defective parvovirus capable of efficiently transfecting respiratory epithelial cells (Flotte et al., 1992; Curiel et al., 1996). Recombinant vectors are stable and appear to have relatively low immunogenicity (Kremer and Perricaudet, 1995). However the carrying capacity of recombinant AAV vectors is small (around 4.7 kb) and the potential for non-site-specific integration (Kremer and Perricaudet, 1995) has raised concerns regarding long-term safety. Furthermore, production of recombinant AAV traditionally requires the presence of a helper virus (usually adenovirus), and practical difficulties exist in generating sufficient yield of recombinant AAV free of contamination with adenovirus or wild-type AAV (Kremer and Perricaudet, 1995). This said, AAV vectors have provided long-term gene expression in animal models, with particular reference to factor IX gene transfer in mice and dogs (Herzog et al., 1999; Snyder et al., 1999). Furthermore a phase I trial in which AAV encoding CFTR was instilled into the sinuses of patients with CF has shown encouraging results (Wagner et al., 1998).

1.7. ADENOVIRAL VECTORS FOR GENE THERAPY

This section will outline the merits of adenovirus as a gene therapy vector, the genomic properties of adenovirus, and the techniques used to generate adenovirus in the laboratory. The problems associated with adenovirus will be discussed with particular reference to host immunity. Finally, novel ways to circumvent the host immune response against adenovirus will be considered.

1.7.1. The merits of adenovirus as a gene therapy vector.

As mentioned above, adenoviruses are such attractive tools for respiratory gene therapy because they have evolved exquisitely efficient mechanisms for the delivery of DNA to the nuclei of respiratory epithelial cells. Recombinant adenoviruses can efficiently infect both dividing and non-dividing cells in a wide

variety of target organs, and do not integrate into the host genome (reviewed by Kremer and Perricaudet, 1995). In addition, adenovirus can be produced in high yield in the laboratory, and is a relatively stable virus *in vivo* (Hitt et al., 1995). Furthermore, adenovirus has proved to be relatively safe in the hundreds of gene therapy protocols in which it has been applied, although this observation has been challenged by important cases in which adenovirus was delivered in very high dose (Crystal et al., 1994; Hollon, 2000).

1.7.2. The structure of adenovirus.

Adenoviruses belong to the family *Adenoviridae*, and are double-stranded DNA viruses. Although several serotypes have been identified, serotypes 2 and 5 are most commonly modified for use in gene therapy protocols. The adenoviral genome is approximately 36 kb in size and comprises two functionally distinct units, the early regions (E1A, E1B, E2A, E2B, E3 and E4, which are transcribed prior to viral replication) and the late regions (L1-5, which are transcribed after viral replication). In general the late regions code for structural proteins. The early regions have a number of functions central to initiating viral transcription, regulating host protein synthesis, and circumventing host immune responses (reviewed by Wold et al., 1994; Kremer and Perricaudet, 1995). The E1 region initiates viral transcription and is the only region which must be transcribed without the aid of *trans* activators encoded by adenovirus (Kremer and Perricaudet, 1995). Viral propagation is thus critically dependent on the E1 region. The implication for gene therapy is that deletion of E1 renders adenovirus replication-deficient whilst freeing space for the insertion of therapeutic transgene.

Wild-type adenovirus capsid takes the form of an icosahedron of approximately 80 nm in diameter. Adherent to the capsid are penton subunits, from which project fibre protein. Elegant studies have demonstrated that the knob of the adenoviral fibre protein engages a specific receptor (the Coxsackie-adenovirus receptor, CAR) on cell surfaces (Bergelson et al., 1997), but that internalisation signals are dependent upon interaction between the penton base and specific cell integrins (Wickham et al., 1993). While these findings significantly advanced understanding of adenovirus biology, there is little doubt that CAR-independent mechanisms of internalisation exist (Hidaka et al., 1999). Once inside cells, efficient mechanisms transport the adenovirus to the nucleus with consequent disassembly

and release of genetic material into an epichromosomal location (Greber et al., 1993; Kremer and Perricaudet, 1995; Greber et al., 1996).

1.7.3. Construction of recombinant adenovirus vectors.

An efficient method for generating recombinant adenoviruses deleted in E1 has been described and developed by Frank Graham's group at McMaster University (Graham and Prevec, 1991; Bett et al., 1994; Hitt et al., 1995). The absolute requirement for E1 in initiating viral transcription is central to this system.

The method, which was used to generate the adenoviruses used in this work (Addison et al., 1997; Sallenave et al., 1998), is illustrated schematically in Figure 5. In brief, two plasmids are used to co-transfect the 293 cell line, an embryonal kidney cell line which can produce E1 *in trans* (Graham et al., 1977). The first plasmid contains all adenoviral sequences required to produce infectious virus when transfected into 293 cells, with the critical exception of the packaging sequence required to encapsidate adenoviral DNA (Bett et al., 1994). The second plasmid is a shuttle plasmid deleted in E1 but containing left-end sequences including a packaging signal, an appropriate promoter, a polylinker sequence, and a further short adenoviral sequence sharing homology with DNA in the first plasmid. The transgene of interest (for example elafin cDNA) is cloned into the polylinker site downstream of the promoter sequence. When the two plasmids are cotransfected into 293 cells, homologous recombination occurs within the short segment common to both plasmids, resulting in an E1 deleted (and thus replication-deficient) construct containing transgene and packaging sequences. As E1 is provided *in trans* by the 293 cell, packaged adenovirus is produced but crucially remains replication-deficient.

With deletion of E1 alone there is space for an insert of approximately 4.7 kb. However the E3 region is not essential for viral replication *in vitro* (Haj-Ahmad and Graham, 1986), and so plasmids have been designed which are also partially deleted in E3, thus freeing up approximately 8.3 kb for insertion of larger transgenes (Bett et al., 1994).

This system is capable of generating high yields of recombinant adenovirus *in vitro*. The stock produced tends to be relatively pure, however for *in vivo* use it is customary to purify 293 cell lysates using CsCl banding, specifically to remove residual eukaryotic proteins and DNA (Hitt et al., 1995). Final precautions include

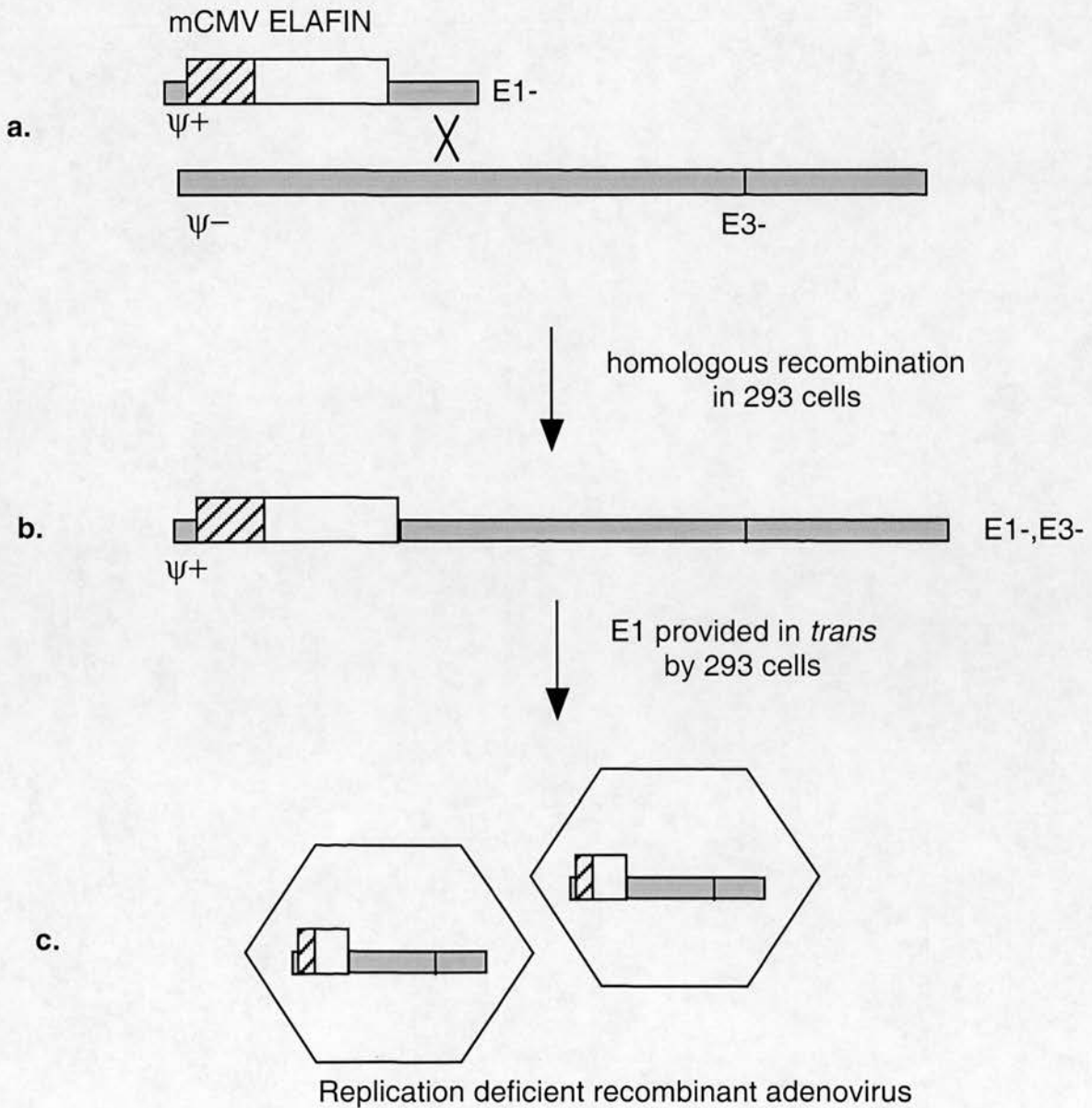


Figure 5. Production of recombinant, replication-deficient adenovirus.

Two complementary circular plasmids are co-transfected into 293 cells (the plasmids are depicted in linear form for convenience). The larger plasmid may be deleted in E3 (E3-) but contains all the sequences required to produce infectious virions in 293 cells, except for the necessary packaging sequence ($\psi-$). The smaller shuttle plasmid contains packaging sequences ($\psi+$) but E1 (which is crucial for viral replication) is deleted (E1-) and replaced with the desired promoter and transgene (in this case the murine cytomegalovirus promoter (mCMV; represented by hatched box) and elafin (represented by open boxes) respectively). Homologous recombination (X) occurs within 293 cells (**a**). This yields viral genome containing packaging sequences and transgene but deleted in E1 and E3 (**b**). 293 cells produce E1 in *trans*, and so viral particles containing transgene are produced, but are themselves E1-deficient (**c**).

dialysis to remove CsCl, and transfection of non-permissive cell lines to exclude the presence of replication competent adenovirus (Fallaux et al., 1999).

1.7.4. Problems associated with the use of recombinant adenoviruses.

A number of theoretical concerns surround the use of recombinant adenoviruses in humans. These include the potential for oncogenicity; the E1 region can confer oncogenicity in rodents (Bernards and van der Eb, 1984), and replication deficient virus can induce inappropriate cyclin expression *in vitro* (Wersto et al., 1998). Furthermore, E1A sequences may be detected in the lungs of healthy individuals and patients with COPD (Matsuse et al., 1992), raising the potential for generation of replication competent virus after administration of E1-deleted adenovirus *in vivo*. In addition, it has been suggested that the apical surface of bronchial epithelium is relatively resistant to transfection with adenovirus (Pickles et al., 1998).

The considerable experience of experimental and clinical pulmonary gene therapy generated in recent years fortunately suggests that these theoretical concerns do not pose significant impediments to successful gene transfer in practice (Kremer and Perricaudet, 1995). However, two very important concerns remain, namely fulminant adenoviral pneumonia (which is extremely rare but may be catastrophic) and host immune responses to adenovirus (which represent the greatest hurdle to be overcome in adenoviral gene therapy).

Severe pneumonia associated with wild-type adenovirus is well recognised and can occur in outbreaks among immunocompetent adults (Klinger et al., 1998). At least two patients treated with high doses of recombinant adenoviruses have had pneumonic illness, one of whom died (Crystal et al., 1994; Hollon, 2000). These tragic events have profound implications for dosage scheduling in future clinical trials.

While adenoviral pneumonia should be readily preventable in future gene therapy protocols, the problem of host immunity to adenovirus is so important as to prevent any form of prolonged transgene expression in clinical practice using adenoviral vectors. The immune response to adenovirus delivered to the lung can broadly be divided into three components. Firstly, innate immune mechanisms sequester a considerable proportion of adenovirus, this largely reflecting rapid

internalisation by alveolar macrophages (Kuzmin et al., 1997; Worgall et al., 1997a). The importance of macrophages to adenoviral gene therapy is returned to in detail in Chapter 4. Secondly, a profound MHC-restricted cytotoxic T lymphocyte response is generated against adenovirus, resulting in florid inflammation and elimination of transfected cells (Yang et al., 1994 and 1996; Wilson, 1996). In recent years evidence has also emerged to implicate CD4+ cells in the early specific response to adenovirus (van Ginkel et al., 1997). The combined effect of innate immunity and the cytotoxic T cell response is to limit significantly the persistence of a single dose of adenovirus. The third immune mechanism, namely the generation of both local and systemic neutralising antibodies to adenovirus (Yei et al., 1994; van Ginkel et al., 1995), significantly reduces the capacity for effective repeat administration. Importantly, the immune response may be directed not only against adenoviral proteins but also against heterologous DNA or protein, a point returned to in Chapter 4 (van Ginkel et al., 1995; Song et al., 1997a; van Ginkel et al., 1997; McLachlan et al., 2000).

A critical point in the study of anti-adenoviral immunity is that the bulk of the evidence has been generated in rodents. Whether the response generated in humans is mediated by similar mechanisms remains controversial. Thus healthy volunteers were noted to have significantly reduced expression of transgene after administration of adenovirus to the lung without a significant rise in neutralising antibodies or in T lymphocyte proliferation to adenoviral antigens (Harvey et al., 2001). Alternative mechanisms of elimination in humans are implied, though the study group was small. The situation in patients with CF has been more fully evaluated but varying results have emerged. For example, generation of anti-adenovirus neutralising antibodies has been described by Zabner et al. (1996) while other groups have found neutralising antibodies to play little if any part in anti-adenoviral immunity (Crystal et al., 1994; Harvey et al., 1999; Zuckerman et al., 1999). In contrast there is no doubt that cell-mediated immunity against adenovirus may be elicited in CF (Crystal et al., 1994; Zuckerman et al., 1999).

1.7.5. Methods available to improve the efficiency of adenoviral gene therapy.

The limitations imposed upon adenoviral gene therapy by host immune responses have generated significant efforts aimed at ameliorating these. In general two strategies have emerged, aimed either at making adenoviral vectors less immunogenic or at co-administration of immunosuppressants.

Recent years have seen the development of "guttled" adenoviruses which contain inverse terminal repeats and packaging signals, but no other sequences encoding viral proteins (Fisher et al., 1996; Kochanek et al., 1996; Parks et al., 1996). These vectors are generated using a helper adenovirus which is deleted of packaging sequences, but which can express structural adenoviral genes in *trans*. These vectors have the distinct advantages of significantly reduced immunogenicity and an increased carrying capacity for large transgenes (Haecker et al., 1996; Chen et al., 1997; Schiedner et al., 1998). Although the requirement for a purification step to remove helper virus partially limits generation of high yields of "guttled" adenovirus, these vectors seem poised to make a significant contribution to adenoviral gene therapy.

Concomitant use of immunosuppressants to deliver adenovirus is potentially less attractive in human subjects. Nonetheless significant advances have been demonstrated in animal models, particularly using techniques to block co-stimulatory molecules involved in immune recognition. Thus transgene expression is enhanced and repeat administration facilitated by blockade of the T cell receptor, by simultaneous administration of ligands blocking CD40 and CD28, and by administration of CTLA4Ig, a chimaeric inhibitor of co-stimulatory molecules (Kay et al., 1995; Wilson and Kay, 1995; Scaria et al., 1997; Zsengellér et al., 1997; Jooss et al., 1998; Wilson et al., 1998). Other strategies which have been employed with success in the laboratory include immune modulation using IL-12 (Yang et al., 1995), induction of immune tolerance using oral administration of adenovirus (Ilan et al., 1997) and administration of immunosuppressant drugs (Vilquin et al., 1995; Kaplan and Smith, 1997). The application of these technologies to humans requires critical evaluation.

In parallel to improvements aimed at curtailing host immune responses, advances may also be expected in the transfection capabilities of adenovirus, and in the power and specificity of gene expression. For example tropism may be expanded by conjugating adenoviral fibre proteins with ligands for cell surface receptors (Rogers et al., 1997). Enhanced expression may be attained through the use of inducible promoters which may be selectively activated (or deactivated) using exogenous chemical "switches" such as tetracycline (Gossen et al., 1995) or ecdysone (No et al., 1996). Interestingly, sequences may be engineered into adenoviral genomes to "insulate" heterologous promoters against the modifying

effects of viral regulatory elements, thus optimising specificity of expression (Steinwaerder and Lieber, 2000). Additionally, tissue-specific and cell-specific promoters may be used to target expression in, for example, type II alveolar epithelial cells and Clara cells (Glasser et al., 1994; Strayer et al., 1998).

1.8. THE APPLICATION OF GENE THERAPY TO RESPIRATORY DISEASE

Respiratory gene therapy has largely evolved from the stimulus to correct the single gene defect of CF. It is instructive to consider CF gene therapy in order to gauge progress in clinical application. CF gene therapy will therefore be considered briefly, followed by the rationale for expanding application of gene therapy to more complex respiratory disorders.

When considering CF gene therapy it is important to acknowledge that conceptual advances have been enormously helped by the development of CFTR "knock-out" mice (reviewed by Grubb and Boucher, 1999) and the subsequent application of gene therapy in these models (Alton et al., 1993). Turning to the situation in humans, a number of small studies have applied the CFTR gene to the nose, sinuses and airways of patients with CF. The vectors employed have been mostly adenoviruses (Zabner et al., 1993; Crystal et al., 1994; Hay et al., 1995; Knowles et al., 1995; Zabner et al., 1996; Bellon et al., 1997; Zuckerman et al., 1999) or cationic liposomes (Caplen et al., 1995; Gill et al., 1997; Porteous et al., 1997; Alton et al., 1999; Hyde et al., 2000) though AAV has also been used (Wagner et al., 1998). In general terms, application of vectors has proved to be safe although studies using adenovirus have been associated with varying levels of inflammation as alluded to earlier (Crystal et al., 1994; Knowles et al., 1995). Proof of principle for transfer of CFTR cDNA to respiratory epithelium has been provided in all studies to date. Importantly, this has been amply demonstrated in the lower respiratory tract for both adenoviral and liposomal vectors (Crystal et al., 1994; Bellon et al., 1997; Alton et al., 1999; Zuckerman et al., 1999). Furthermore some studies have demonstrated partial functional correction of the electrophysiological phenotype characteristic of CF (Zabner et al., 1993; Caplen et al., 1995; Hay et al., 1995; Alton et al., 1999), though this has not been a universal finding (Knowles et al., 1995). Unfortunately all studies to date have been characterised by a disappointingly low proportion of cells transduced, and by short-lived expression of CFTR.

Perhaps the most detailed and encouraging study so far was a randomised, placebo-controlled, double blind trial using nebulised liposomes encoding CFTR (Alton et al., 1999). Although the study recruited patients with relatively early impairment of lung function, and despite transient side effects being common, the trial demonstrated a significant improvement in lower airway potential difference as well as a reduction in bacterial adherence in the treated group (Alton et al., 1999). Thus while CF gene therapy is a very considerable way from effecting lasting benefit, significant advances have been made. Basic research in CF knock-out mice continues to provide tantalising leads for clinical gene therapy as exemplified by the observation that only a small proportion of CF cells need be transfected with CFTR to effect phenotypic correction (Dorin et al., 1996), and the observation that a single intra-amniotic administration of adenovirus encoding CFTR can reverse the CF phenotype (Larson et al., 1997).

To some extent the study of CF exemplifies the advances and continued challenges in gene therapy. However gene therapy has expanded its horizons well beyond monogenic disorders such as CF and α_1 -PI deficiency, and clinical applications are being developed particularly for lung cancer and mesothelioma (reviewed by Simpson et al., 2000). Indeed the potential use of gene therapy is being investigated in a plethora of increasingly complex respiratory conditions in the laboratory, and it is against this background that the experiments described in this thesis were performed.

1.9. SUMMARY

This introduction has outlined the importance of the neutrophil in acute pulmonary inflammation, and has suggested a central role for HNE in acute inflammatory injury. Consideration of endogenous inhibitors of HNE has suggested an important role for elafin, a low molecular weight, cationic molecule which is up-regulated by early inflammatory cytokines. Augmentation of endogenous inhibitors of HNE appears to be an attractive strategy for the attenuation of neutrophil-mediated lung injury. Furthermore, the rationale for using adenovirus to target expression of protective molecules in the lung has been presented. These principles generated the hypotheses central to this work.

1.10. CENTRAL HYPOTHESES

The initial hypothesis driving this work was that ***genetic augmentation of host defence molecules in pulmonary epithelial cells would result in protection against damage mediated by acute inflammatory stimuli both in vitro and in complex in vivo models of lung injury***. The model used to test this hypothesis *in vitro* centred around the transfection of A549 cells (derived from alveolar epithelium) with adenovirus encoding human elafin cDNA, with subsequent exposure of the cells to HNE or activated human neutrophils. The data generated by the subsequent experiments are described in Chapter 3.

Before extending these principles to study models of lung *injury*, it was first considered important to assess the effect of elafin gene augmentation on neutrophil migration to the lung, in view of the known influence of elastase on neutrophil extravasation. Thus a further principal hypothesis was that ***elafin gene augmentation in vivo would influence the neutrophil influx characteristic of pulmonary inflammation***. The model chosen to test this hypothesis involved transfection of murine airways with adenovirus encoding human elafin cDNA, followed by IT challenge with bacterial LPS. The results arising from this model are described in Chapter 4.

The findings in Chapter 4, in conjunction with elafin's properties as a low molecular weight, cationic inhibitor of HNE expressed predominantly at epithelial sites and up-regulated in response to early inflammatory cytokines, stimulated the further hypothesis that ***elafin has intrinsic antimicrobial activity***. This was tested by applying elafin (and its structural domains) to *Pseudomonas aeruginosa* and *Staphylococcus aureus in vitro*. The results are presented in Chapter 5.

Data generated by experiments in Chapters 3, 4 and 5 were ultimately used to test the key hypothesis that ***elafin gene augmentation could protect the pulmonary epithelium in in vivo models of acute inflammatory lung injury***. The model employed involved transfection of murine airways with adenovirus encoding human elafin, followed by challenge with IT *Pseudomonas aeruginosa*. Results from this model are described in Chapter 6.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Reagents prepared specifically for studies of elafin biology.

2.1.1.1. Adenoviral constructs.

Two E1-, partially E3-deleted adenoviral constructs of serotype 5 were used. Details of their preparation are described below and followed the principles outlined in section 1.7.3. These vectors were available in-house from the outset of this work. The first (Ad-elafin) consists of human elafin cDNA downstream of the mCMV promoter, and was constructed by Dr Jean-Michel Sallenave in Professor Jack Gauldie's laboratory at McMaster University, Hamilton, Ontario, Canada (Sallenave et al., 1998). The second was used throughout this work as a control vector, and contains the lacZ reporter gene downstream of the mCMV promoter (Ad-lacZ). Ad-lacZ was originally constructed by Dr Christina Addison and her colleagues in Professor Frank Graham's laboratory at McMaster University (Addison et al., 1997). Amplification and purification of the batches used in this study were performed by Dr Sallenave, Mr Duncan Chong and Mrs Xueya Feng in Professor Gauldie's laboratory at McMaster University. The techniques used are reviewed by Hitt et al. (1995).

For the preparation of Ad-elafin, full-length human elafin cDNA (538 bp) was obtained by reverse transcriptase polymerase chain reaction (RT-PCR) from total RNA extracted from the bronchial epithelial cell line NCI-H322, which shares phenotypic characteristics with Clara cells and is known to secrete elafin (Sallenave et al., 1993). As described by Sallenave et al. (1998) elafin cDNA was ultimately cloned into a shuttle plasmid (pDK6) containing Ad5 sequences from 0-1 mu and 9.8-15.8 map units (mu), a polyadenylation signal, and a 1.4 kb fragment of the mCMV immediate early promoter (Dorsch-Häsler et al., 1985). The polylinker site into which elafin cDNA was cloned is downstream of the mCMV promoter. The resulting plasmid was cotransfected with pBHG10 in 293 cells (Sallenave et al., 1998). PBHG10 lacks a packaging sequence and is partially E3-deleted (ie it contains the entire Ad5 gene sequence apart from 0.5-3.7 mu and 78-85.6 mu).

Homologous recombination resulted in E1-, partially E3-deleted adenovirus encoding elafin cDNA downstream of the mCMV promoter. The resulting Ad-elafin vector was purified as described below.

Ad-lacZ was prepared using the same principles. In brief, Ad-lacZ was cloned into plasmid pMH5 (from which pDK6 was derived) downstream of the same 1.4 kb mCMV promoter fragment (Dorsch-Häsler et al., 1985). The resulting plasmid was cotransfected with pBHG10 in 293 cells (Addison et al., 1997).

With reference to purification and titering of both vectors, crude 293 cell lysates were prepared and added to 293N3S cells in spinner culture flasks. After 3 to 4 days the presence of inclusion bodies was observed in at least 90% of cells. Cells were pelleted, lysed then "banded" using step/continuous CsCl gradients. The adenovirus band was collected and further desalted using PD-10 column chromatography.

2.1.1.2. Recombinant human elafin.

A truncated recombinant human elafin (57 amino acids in length, H₂N-³⁹AQE.....⁹⁵Q-OH, approximately 6 kDa, representing the COOH-terminus of pro-elafin) was available in-house, and was originally a kind gift from Dr J Fitton, ICI Pharmaceuticals, Macclesfield, UK.

2.1.1.3. Synthetic human elafin peptides.

Elafin peptides were prepared by Albachem (Edinburgh, UK) using standard techniques described elsewhere (Morrison et al., 1998). The established gene sequence for human elafin (Sallenave and Silva, 1993) was used to derive amino acid sequences. Four peptides were supplied, namely

- Folded full-length elafin (H₂N-¹AVT.....⁹⁵Q-OH), molecular weight 9925 Da.
- Unfolded full-length elafin.
- Folded NH₂-terminal domain (H₂N-¹AVT.....⁵⁰K-OH), molecular weight 5172 Da.
- Folded COOH-terminal domain (H₂N-⁵¹PGS.....⁹⁵Q-OH), molecular weight 4776 Da.

The molecular mass of each moiety was determined by mass spectrometry (Albachem) and corresponded to the predicted mass in each case.

The amino acids demarcating the end of the NH₂-terminal domain and the start of the COOH-terminal domain were determined from the known crystal structure of a fragment of elafin complexed to PPE (Tsunemi et al., 1996), and from the known structure and sequence of human SLPI (Grütter et al., 1988).

2.1.1.4. Antiserum raised in rabbits against human elafin.

Antiserum raised against a COOH-terminal 2.5 kDa fraction of human elafin was prepared by Dr Jean-Michel Sallenave as described elsewhere (Sallenave et al., 1992), and was available in-house from the outset of this work.

2.1.1.5. Bacteria.

Pseudomonas aeruginosa strain PAO1 (an extensively characterised clinical strain (reviewed by Govan and Deretic, 1996; Stover et al., 2000)), and *Staphylococcus aureus* strain C1705 (Morrison et al., 1998) were available in-house, and were kindly provided by Professor John Govan, Department of Medical Microbiology, University of Edinburgh.

2.1.2. Source of other reagents used.

All reagents were from Sigma, Poole, Dorset, UK, with the exception of those listed below.

L-glutamine, penicillin-streptomycin, trypsin-ethylene diamine tetraacetic acid (EDTA), Trizol, and Iscove's medium were from Gibco BRL, Paisley, Scotland, UK. 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) was from Promega, Madison, USA.

¹¹¹Indium was from Dupont, NEN Life Sciences, Brussels, Belgium.

Dextran and Percoll were from Pharmacia, Uppsala, Sweden.

HNE was from Elastin Products, Owensville, MO, USA.

PAF and fMLP were from Calbiochem, Nottingham, UK.

Tetramethylbenzidine (TMB) was from Boehringer Mannheim, Mannheim, Germany.

Gelatin, avidin-biotin and avidin-biotin buffer were from BDH, Poole, Dorset, UK.

LPS (from *Escherichia coli* 0127:B8 in all experiments described), yeast extract and *Pseudomonas* Isolation Agar (PIA) were from Difco Laboratories, Detroit, MI, USA.

Nitrocellulose membrane was from Millipore, Watford, UK.

Dried skimmed milk was used in the form of Marvel, Premier Brands UK, Moreton, UK.

Murine anti-rabbit immunoglobulin complexed to peroxidase and 3,3'-diaminobenzidine tetrachloride (DAB) were from DAKO, Glostrup, Denmark. Enhanced chemiluminescence (ECL) kit was from Amersham Pharmacia, Little Chalfont, UK.

X-omat radiographic film was from Kodak, Chalon-sur-Saône, France.

Tryptone soya broth (TSB), nutrient agar and Columbia agar were from Unipath, Basingstoke, UK.

Recombinant human SLPI, and enzyme-linked immunosorbent assay (ELISA)s for human IL-1, IL-8, human TNF- α , murine TNF- α and macrophage inflammatory protein 2 (MIP-2) were from R&D Systems, Abingdon, UK..

2,2,2-tribromoethanol was from Aldrich, Gillingham, UK.

Nutrient broth was from Oxoid, Basingstoke, UK.

Assay of protein concentration, and NHS-LC-Biotin kit were from Pierce, Rockford, IL, USA.

Murine albumin ELISA was from Bethyl Laboratories, Montgomery, TX, USA.

Diff-quick stain was from Dade Diagnostika, Germany.

Normal goat serum was from the Scottish Antibody Production Unit, Edinburgh.

2.1.3. Plastic-ware.

24-well tissue culture plates and Transwell polycarbonate filters were from Corning Costar, Cambridge, MA, USA.

Plates for elafin ELISA were from Linbro, Flow Laboratories, McLean, VA, USA.

Nylon tubing for IT instillation was from Portex, Hythe, Kent.

2.1.4. Mice.

Female C57/Bl6 mice aged between 6 and 8 weeks were provided by Harlan Olac, Bicester, UK.

2.2. METHODS

2.2.1. Preparation of human serum, neutrophils and peripheral blood-derived mononuclear cells.

The method used followed that described by Haslett et al. (1985). Fresh whole blood was obtained from healthy volunteers and added to sodium citrate (final concentration 0.38%). 50ml aliquots of blood in plastic tubes were centrifuged at 350g for 20 minutes at room temperature to yield platelet rich plasma (PRP) above a red cell pellet. PRP was aspirated and decanted into glass tubes; autologous serum was prepared by adding CaCl_2 and incubating at 37°C. Immediately after aspiration of PRP, dextran was gently mixed with the red cell pellet (0.25ml dextran per ml of red cell pellet) and incubated at room temperature for 30 minutes to allow sedimentation. The uppermost, leukocyte rich layer was gently aspirated, and saline added to a final volume of 50ml. The suspension was centrifuged at 350g for 6 minutes at room temperature yielding a white cell pellet which was gently resuspended in 2.5ml of 55% Percoll (prepared in phosphate buffered saline (PBS)). The suspension was gently layered onto 2.5ml of 70% Percoll, which had in turn been gently layered onto 2.5ml of 81% Percoll (ie forming a sequential Percoll density gradient, cells being suspended in the uppermost layer). The Percoll gradient was centrifuged at 700g for 20 minutes at room temperature. This results in peripheral blood-derived mononuclear cells (PBMCs) being suspended in a band at the interface between 55% and 70% Percoll, while polymorphonuclear leukocytes exist in a band between 70% and 81% Percoll. Each band was gently aspirated and washed twice with PBS, each wash being followed by centrifugation at 220g for 5 minutes at room temperature. The resultant pellets were each suspended in PBS. Small aliquots were taken from each for quantification using a haemocytometer, for assessment of cell morphology and cell type by flow cytometry, and for differential cell count by preparation of cytopins stained using Diff-quick. Neutrophil preparations were deemed satisfactory for further analysis if >95% of the polymorphonuclear band consisted of neutrophils.

Neutrophils and PBMCs were used immediately after preparation. If the subsequent experimental protocol required that cells be suspended in medium other than PBS, cells were centrifuged again at 220g for 5 minutes at room temperature, PBS discarded, and cells suspended at known concentration in the medium of choice.

2.2.2. Preparation of A549 cells.

A549 cells (Giard et al., 1973) were available in-house. Cells were suspended in Dulbecco's Modified Eagles Medium (DMEM) containing 10% foetal calf serum (FCS), penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100µg/ml) and L-glutamine (final concentration 2µM), then seeded onto 24-well plastic tissue culture plates and incubated at 37°C in a humidified incubator containing 5%CO₂. Cells were used in further experiments only once full confluence was achieved.

Estimation of the number of cells in each confluent monolayer was performed by washing cells with PBS then adding trypsin and incubating at 37°C. Cells were agitated to obviate clumping, and detachment was confirmed by light microscopy. Cells were counted using a haemocytometer. Cell viability was assessed by staining with trypan blue, with exclusion indicating viability. Standard 24-well plastic plates were used, and at confluence each well contained approximately 300,000 A549 cells.

2.2.3. Transfection of A549 cells with adenoviral constructs.

Adenoviral constructs of known concentration were diluted in DMEM containing 5% FCS, penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100µg/ml) and L-glutamine (final concentration 2µM). A549 cells were washed with PBS and adenovirus applied at a multiplicity of infection (moi) of 0-50 plaque forming units (pfu) (total volume 100µl per well) for 30 minutes at 37°C. Cells were washed repeatedly with PBS to remove excess virus and incubated overnight at 37°C in DMEM containing 10% FCS, penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100µg/ml) and L-glutamine (final concentration 2µM). The following day, except where otherwise stated, cells were washed to remove all traces of serum, and 400µl of fresh serum-free DMEM containing penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100µg/ml) and L-glutamine (final concentration 2µM) was added. The rationale for using serum-free medium stems from the fact that serum contains a high concentration of elastase inhibitors, and many of the experiments described were designed specifically to determine whether Ad-elafin could confer protection against the effects of HNE.

Experiments assessing the effects of elafin augmentation were performed using Ad-elafin to stimulate elafin expression, Ad-lacZ as a viral control, and vehicle alone as a non-viral control.

2.2.4. Staining for β -galactosidase in A549 cells.

A549 cells, transfected as described in section 2.2.3., were washed with PBS. Serum-free DMEM containing penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100 μ g/ml) and L-glutamine (final concentration 2 μ M) was added and the cells incubated at 37°C for 24 hours. Medium was discarded and 200 μ l of fixative added for 10 minutes at room temperature (fixative comprised 0.2% glutaraldehyde, 0.8% formaldehyde, 2mM MgCl₂, in PBS). Fixative was discarded and 200 μ l of staining solution added for 5 hours at 37°C (staining solution comprised 5mM K₄Fe(CN)₆, 5mM K₃Fe₃(CN)₆, 2mM MgCl₂, 0.05% Triton X-100, 0.5 mg/ml X-gal, in PBS). Cells were washed with PBS and photographs were taken.

2.2.5. Labelling of A549 cells with ¹¹¹Indium.

The technique used was adapted from that described for radiolabelling of human endothelial cells (Smedley et al., 1986). Radiolabelled indium was incubated with 4x10⁻³M tropolone for 1 minute at room temperature (final concentration of ¹¹¹Indium 75 μ Ci/ml). 40 μ l of the resulting solution (3 μ Ci of ¹¹¹Indium, final concentration of tropolone 4x10⁻⁴M) was added directly to transfected (and untransfected) A549 cells at the end of the overnight incubation in DMEM containing 10% FCS. Incubation proceeded for 15 minutes at room temperature. Supernatants were gently aspirated, and cells were washed three times with PBS. 400 μ l of fresh, serum-free DMEM containing penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100 μ g/ml) and L-glutamine (final concentration 2 μ M) was added to each well in keeping with the scheme outlined in section 2.2.3.

In each experiment performed 40 μ l of the ¹¹¹Indium solution applied directly to cells (ie 3 μ Ci of ¹¹¹Indium in 4x10⁻³M tropolone) was added to scintillation fluid and radioactivity measured in a scintillation analyser. The supernatant and three washes retrieved from representative wells were also added to scintillation fluid and assessed for radioactivity; the sum of radioactivity from these aliquots was

subtracted from that in the initial $^{111}\text{Indium}$ solution. Incorporation of $^{111}\text{Indium}$ was estimated by dividing the resulting radioactivity (assumed to be incorporated) by that in the initial $^{111}\text{Indium}$ solution.

Further confirmation of incorporation was provided by lysing representative monolayers using Tryzol, then estimating radioactivity in the resulting suspensions.

2.2.6. Assessment of damage to A549 cells induced by addition of HNE or activated human neutrophils.

A schematic representation of the experiments performed is shown in Figure 1. A549 cells transfected as described in section 2.2.3. were washed with PBS to remove serum, and 400 μl of serum-free DMEM containing penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100 $\mu\text{g}/\text{ml}$) and L-glutamine (final concentration 2 μM) were added to each monolayer. Cells were incubated at 37°C for 24-72 hours depending on the experimental conditions required. HNE was added directly at a final concentration ranging from 0-4 $\mu\text{g}/\text{ml}$ and cells were incubated for a further 16 hours at 37°C. Damage to the monolayer was assessed morphologically by light microscopy, and quantified by aspirating and agitating the supernatant (to obviate cell clumping), staining with trypan blue, and counting cells using a haemocytometer. In a variation of these experiments A549 cells were radiolabelled with $^{111}\text{Indium}$ as described above and damage to the monolayer quantified by measuring radioactivity in supernatants using a scintillation analyser.

In a separate set of experiments the same protocol was employed with the exception that activated human neutrophils substituted for HNE. Neutrophils were prepared fresh from whole blood as described in section 2.2.1. and suspended in serum-free DMEM containing penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100 $\mu\text{g}/\text{ml}$) and L-glutamine (final concentration 2 μM). 40 μl of neutrophil suspension (incorporating either 1×10^6 , 1.5×10^6 or 2×10^6 neutrophils in different experiments) was added directly to wells containing A549 cells which had been incubated for 24-72 hours (depending on the experimental conditions required) at 37°C in 400 μl of serum-free DMEM containing penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100 $\mu\text{g}/\text{ml}$) and L-glutamine (final concentration 2 μM). Immediately upon addition of neutrophils, 1% bovine serum albumin (BSA) (final concentration) was added to

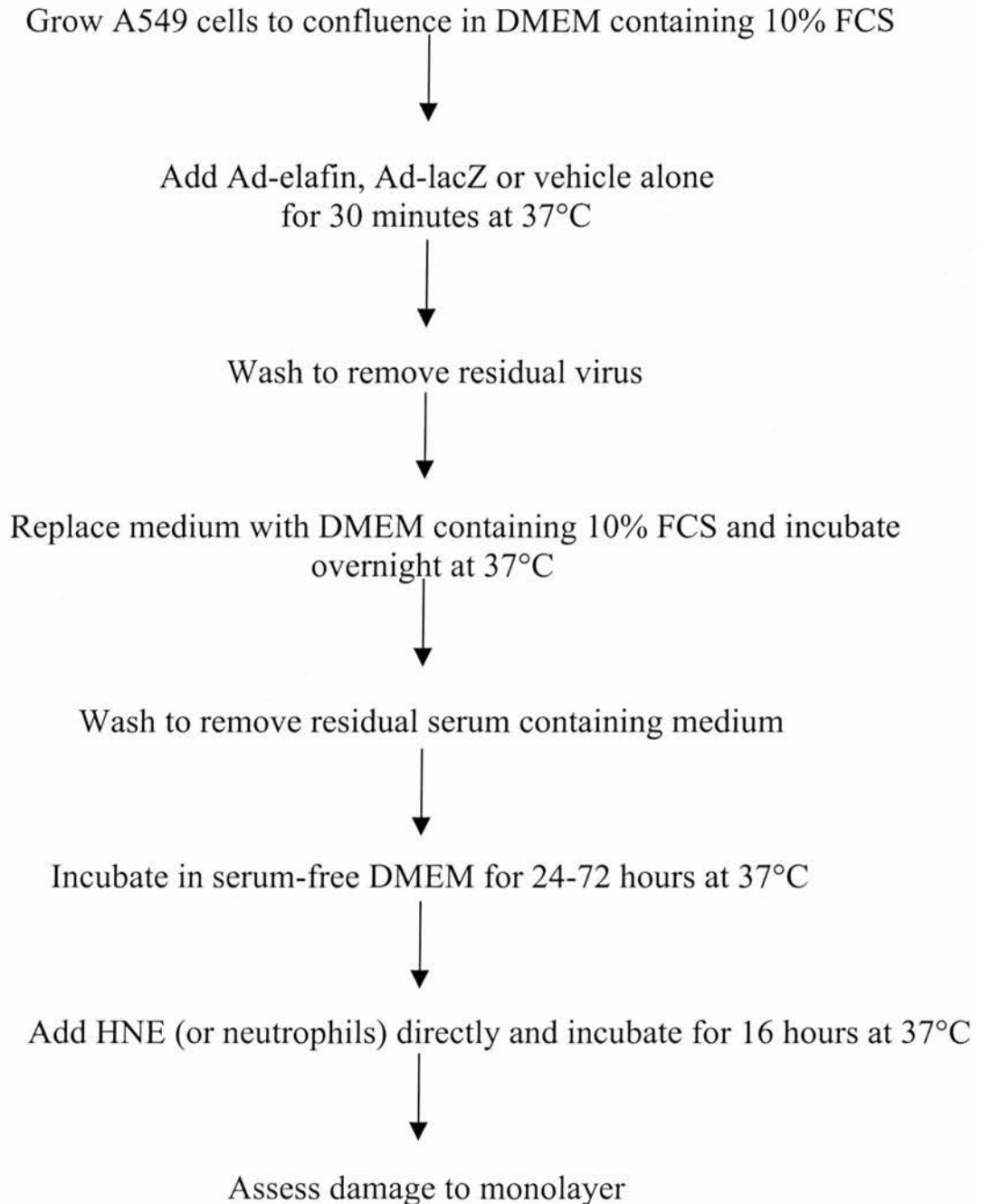


Figure 1. Outline of the principal stages of experiments assessing the effect of Ad-elafin on A549 cell damage mediated by HNE or activated human neutrophils.

all wells. At this point neutrophils were stimulated in one of two ways. In the first phorbol myristate acetate (PMA) was added (final concentration 1 ng/ml). In the second the priming agent PAF was added (final concentration 10^{-9} M) for 1 hour at 37°C followed by the addition of fMLP (final concentration 10^{-7} M). The concentrations of PAF and fMLP described were determined from preliminary experiments using PAF in the dose range 10^{-9} M to 10^{-11} M, and fMLP in the range 10^{-7} M to 10^{-9} M, optimal degranulation of human neutrophils occurring with the combination of 10^{-9} M PAF and 10^{-7} M fMLP. Irrespective of the neutrophil stimulant used, incubation proceeded at 37°C . 16 hours after the addition of neutrophils, damage to the monolayer was assessed morphologically using light microscopy.

In a variation of these experiments, A549 cells were radiolabelled with $^{111}\text{Indium}$ as described above, and neutrophils stimulated using PAF (10^{-9} M) and fMLP (10^{-7} M). Damage to the monolayer was assessed by measuring radioactivity in cell supernatants at the end of the experiment. To control for the theoretical possibility of non-specific leak of radiolabel from A549 cells, radioactivity in supernatants from cells not exposed to neutrophils was subtracted from that in supernatants from cells exposed to activated neutrophils.

2.2.7. Rabbit anti-human elafin polyclonal antibody; preparation, confirmation of activity against elafin, and addition to A549 cells.

Immune serum raised against human elafin in rabbits (Sallenave et al., 1992) was passed over a protein A-sepharose column, and the column washed with 0.1M Tris, pH 8.0. Elution was performed using 0.1M glycine, pH 3.0, buffered with 1M Tris, pH 8.0. The concentration of the eluent was established by measuring absorbance at 280nm.

Varying concentrations of the eluted fraction were incubated with 100ng of recombinant human elafin for 30 minutes at 37°C . HNE (0-1200ng) was then added, with further incubation for 30 minutes at 37°C . The final volume of the elafin/anti-elafin/HNE mixture was 50 μl . Elastase activity was measured as described in section 2.2.12.

Upon determination of the concentration of anti-elafin antibody required to neutralise 2 ng/ μl of elafin, an excess of anti-elafin antibody was added to transfected A549 cells which had been incubated for 24 hours in serum-free DMEM

containing penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100µg/ml) and L-glutamine (final concentration 2µM). HNE (4 µg/ml final concentration) was added directly to supernatants and damage to the A549 monolayer was assessed 16 hours later by light microscopy (as described in section 2.2.6. above). Supernatants were retrieved for measurement of residual elastase activity as described in section 2.2.12.

2.2.8. Neutrophil migration assay.

A549 cells were transfected as described in section 2.2.3. Cells were washed with PBS to remove residual serum, and medium replaced with 400µl of serum-free phenol red-free (PRF)-DMEM containing penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100µg/ml) and L-glutamine (final concentration 2µM). Cells were incubated for 48 hours at 37°C. 1% BSA (final concentration) was then added directly to each well.

Human neutrophils were prepared as described in section 2.2.1. and suspended in PRF-DMEM containing 1% BSA (final concentration), penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100µg/ml) and L-glutamine (final concentration 2µM). 100µl of the suspension (containing 1.25×10^6 neutrophils) was added to polycarbonate filters of 3µm pore size. Filters were immediately placed into wells containing A549 cells, such that the base of the filter was just in contact with A549 cell supernatant. Incubation proceeded at 37°C for 20 minutes, at which point filters were removed and discarded. Lysis of neutrophils which had migrated through the filter was then achieved by direct addition of Triton X-100 (final concentration 0.4%). The resulting lysate was acidified using 12.5µl of Na citrate-acetate buffer, pH4.2.

MPO activity was used as an index of neutrophil content and was measured by adding 100µl of MPO-specific chromogenic substrate (0.1 mg/ml TMB, 0.03% H₂O₂, in 0.1M Na acetate-citrate, pH 4.9) to 100µl of lysed supernatant and quantifying the change in absorbance at 630nm as a function of time. The number of neutrophils in lysates was estimated by extrapolation from standard curves prepared by lysing known quantities of neutrophils and plotting against the change in absorbance at 630nm after addition of TMB and H₂O₂. Because the rate of migration of neutrophils from different donors varied considerably (89,000-299,000, median 237,000, n=4) neutrophil migration was expressed relative to maximal migration for

each donor. PRF-DMEM was used in these experiments because the blue colour generated by addition of chromogenic substrate is poorly detected in medium containing phenol red.

A number of variations of this experiment were performed. In the first, an excess of anti-elafin immunoglobulin was added to cells prior to the addition of neutrophil-containing inserts. In the second, recombinant human elafin was added to cell-containing and cell-free wells prior to the addition of inserts. In the third anti-human-IL-8 was added to cells prior to the addition of inserts.

2.2.9. Addition of LPS to A549 cells.

A549 cells were transfected as described in section 2.2.3.. After incubation in DMEM containing 10% FCS, penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100µg/ml) and L-glutamine (final concentration 2µM) for 48 hours, LPS was added directly to cells at a final concentration ranging between 0 and 1000 µg/ml (ie in experiments using LPS serum was present in supernatants throughout unless otherwise stated). Cells were incubated for a further 24 hours at 37°C and supernatants were retrieved. In a variation of these conditions LPS was added immediately after transfection at the first point of exposure to DMEM containing 10% FCS, penicillin G (final concentration 100 U/ml), streptomycin sulfate (final concentration 100µg/ml) and L-glutamine (final concentration 2µM). Cells were incubated for 72 hours at 37°C and supernatants were retrieved.

The viability of A549 cells treated with LPS was assessed by aspirating supernatants from representative wells, staining with trypan blue and assessing the proportion of viable cells using a haemocytometer. The remaining monolayers were washed with PBS and treated with trypsin to detach cells. Cells were agitated to prevent clumping, stained with trypan blue, and assessed using a haemocytometer.

2.2.10. Elafin ELISA.

The ELISA was performed on 96 well plastic plates. Wells were coated overnight at 4°C with anti-elafin IgG in carbonate buffer, pH 9.6 (final concentration IgG 7.7 µg/ml), with the exception that "blank" wells received buffer alone. The plate was washed with wash buffer (0.1% Tween-20 in PBS, pH 7.4).

Gelatin (final concentration 1% in PBS, pH 7.4) was added to all wells (as a blocking agent) for 2 hours at 37°C and the plate washed with wash buffer, prior to the addition of samples and standard.

Standards comprised recombinant human elafin diluted in 1% gelatin, and were applied in triplicate at a dose range from 0.5 to 30 ng/ml ("blanks" received 1% gelatin alone). Samples were serially diluted in 1% gelatin; all dilutions were applied in duplicate. The volume of each standard and sample was 100µl, and incubation proceeded for 2 hours at 37°C, after which all wells were washed with wash buffer. Anti-elafin IgG, biotinylated using an NHS-LC-Biotin kit, was diluted in 1% gelatin and added to each well (except "blanks" which received 1% gelatin alone) for 2 hours at 37°C. The plate was washed with wash buffer.

Streptavidin-biotin-horseradish peroxidase complex (final concentration 10 ng/ml in 1% gelatin) was added to each well (except "blanks" which received 1% gelatin alone) for 2 hours at 37°C. The plate was washed with wash buffer. Substrate (comprising 0.006% H₂O₂ and 1 mg/ml 2,2' azino bis 3-ethyl benzthiazolidine sulfonic acid, in 0.1M citric acid, pH 4.0) was added to all wells and absorbance read at 550nm. Standard curves were constructed by plotting absorbance against elafin concentration, and the elafin concentration in samples determined by extrapolation from the curve.

2.2.11. "Dot-blot" detection of elafin.

Samples of conditioned medium from transfected A549 cells were applied directly to a nitrocellulose membrane at room temperature and allowed to dry. PBS (as a negative control) and recombinant human elafin at a final concentration of 0.1 or 1 µg/ml (as positive control) were also applied. The membrane was added to an excess of blocking solution, comprising 5% dried skimmed milk and 0.05% Tween 20 in PBS. Incubation proceeded overnight at 4°C with continuous orbital shaking.

The blocking solution was discarded and anti-elafin IgG (diluted one thousand-fold in blocking solution, final volume 10ml) was added for 1 hour at room temperature with continuous orbital shaking. The membrane was washed with an excess of PBS-Tween (0.05% Tween 20 in PBS).

Murine anti-rabbit immunoglobulin conjugated to peroxidase (diluted five thousand-fold in blocking solution) was then added for 20 minutes at room temperature with continuous orbital shaking. The membrane was washed with blocking solution for 15 minutes at room temperature, and then washed with PBS-Tween. Chemiluminescence was developed using a standard ECL kit, and the membrane developed on X-omat radiographic-quality film.

2.2.12. Assessment of elastase activity.

Equal volumes (50 μ l unless otherwise stated) of sample and elastase-specific substrate were added to 96 well plastic plates. Substrate consisted of 0.1 mg/ml N-methoxysuccinyl-ala-ala-pro-val p-nitroanilide in 50mM Tris, 0.1% Triton, 0.5M NaCl, pH 8.0. Absorbance at 405nm was measured serially, immediately from the time of addition of substrate. Absorbance was plotted against time and, within the linear portion of the plot, change of absorbance was measured.

In all cases blanks consisted of equivalent volumes of substrate and the unconditioned medium in which the experiment was performed (for example when measuring elastase activity in supernatant from A549 cells incubated in DMEM to which HNE was added, blanks consisted of DMEM and substrate).

When considering samples in which the concentration of elastase was unknown (for example after addition of neutrophils), experiments included a well in which purified HNE replaced sample, and acted as a standard.

When considering samples in which the concentration of elastase was pre-determined (for example where HNE had been added to A549 cell conditioned medium to give a final concentration of 4 μ g/ml), elastase activity in supernatants from transfected cells was expressed relative to the elastase activity in supernatants from untransfected cells.

2.2.13. Measurement of elastase inhibitory activity (EIA).

The principles of measurement of EIA are an extension of those for measurement of elastase activity.

Samples were serially diluted in 50mM Tris, 0.1% Triton, 0.5M NaCl, pH 8.0. 10µl of each dilution was added to 50ng of HNE in 10µl of 50mM Tris, 0.1% Triton, 0.5M NaCl, pH 8.0. The final volume was made up to 50µl with 50mM Tris, 0.1% Triton, 0.5M NaCl, pH 8.0. Plates were incubated for 30 minutes at 37°C. In all experiments one solution consisted of 10µl HNE and 40µl 50mM Tris, 0.1% Triton, 0.5M NaCl, pH 8.0 (ie no sample).

To each sample/HNE/buffer mix of 50µl was added 50µl of elastase substrate (0.1 mg/ml N-methoxysuccinyl-ala-ala-pro-val p-nitroanilide in 50mM Tris, 0.1% Triton, 0.5M NaCl, pH 8.0). Absorbance at 405nm was plotted against time and, within the linear portion of the plot, change of absorbance was measured. Change of absorbance was then plotted against volume of sample. On the assumption that HNE forms 1:1 stoichiometric complexes with its natural inhibitors in samples generated *in vitro* and *in vivo*, extrapolation of the resultant curve to the abscissa indicates the volume of sample which completely inhibits the known starting concentration (0.5ng/µl) of HNE (after Bieth, 1980). The molar concentration of elastase inhibitors in the sample (ie the EIA) can then be calculated.

2.2.14. Culture of monocyte-derived macrophages.

PBMCs were freshly prepared as described in section 2.2.1., and suspended in serum-free Iscove's medium containing penicillin G (final concentration 100 U/ml) and streptomycin sulfate (final concentration 100µg/ml) at a concentration of approximately 4×10^6 cells/ml. The suspension was added to 24-well plastic tissue culture plates and incubated for 1 hour at 37°C. Non-adherent cells were washed off with warm serum-free Iscove's medium containing penicillin G (final concentration 100 U/ml) and streptomycin sulfate (final concentration 100µg/ml). Medium was then changed to Iscove's medium containing 10% heat inactivated autologous serum (HIAS), penicillin G (final concentration 100 U/ml) and streptomycin sulfate (final concentration 100µg/ml). Cells were incubated at 37°C. After 3 days cells were washed again with addition of fresh Iscove's medium containing 10% HIAS, penicillin G (final concentration 100 U/ml) and streptomycin sulfate (final

concentration 100µg/ml). This protocol selects out monocytes, as lymphocyte populations tend to be non-adherent. Under these conditions monocytes differentiate to a macrophage phenotype within approximately 5 days of collection (Wright et al., 1983). Cells were therefore transfected either 5 or 6 days from the time of obtaining PBMCs.

2.2.15. Transfection of monocyte-derived macrophages with adenoviral constructs.

The principles of transfection are as described for transfection of A549 cells (section 2.2.3.). Adenoviral constructs of known concentration were diluted in Iscove's medium containing 5% HIAS, penicillin G (final concentration 100 U/ml) and streptomycin sulfate (final concentration 100 µg/ml). Monocyte-derived macrophages were washed with PBS and adenovirus applied at an moi of 0-100 pfu (total volume 100µl per well) for 30 minutes at 37°C. Cells were washed with PBS to remove excess virus and incubated overnight at 37°C in Iscove's medium containing 10% HIAS, penicillin G (final concentration 100 U/ml) and streptomycin sulfate (final concentration 100 µg/ml).

2.2.16. Staining of monocyte-derived macrophages for β -galactosidase.

The principles of β -galactosidase staining are as described in section 2.2.4. Monocyte-derived macrophages were transfected as described in section 2.2.15. and incubated at 37°C for 48 hours. Medium was discarded and 200µl of fixative added for 10 minutes at room temperature (fixative comprised 0.2% glutaraldehyde, 0.8% formaldehyde, 2mM MgCl₂, in PBS). Fixative was discarded and 200µl of staining solution added for 5 hours at 37°C (staining solution comprised 5mM K₄Fe(CN)₆, 5mM K₃Fe₃(CN)₆, 2mM MgCl₂, 0.05% Triton X-100, 0.5 mg/ml X-gal, in PBS). Cells were washed with PBS and photomicrographs were taken.

2.2.17. Addition of LPS to monocyte-derived macrophages.

The principles of LPS stimulation are as described in section 2.2.9. Monocyte-derived macrophages were transfected as described in section 2.2.15. After incubation in Iscove's medium containing 10% HIAS, penicillin G (final concentration 100 U/ml) and streptomycin sulfate (final concentration 100 µg/ml) for 48 hours, LPS was added directly to cells at a final concentration ranging

between 0 and 1000 µg/ml. Cells were incubated for a further 24 hours at 37°C and supernatants retrieved.

In one variation of this experiment, LPS was added immediately upon addition of Iscove's medium containing 10% HIAS, penicillin G (final concentration 100 U/ml) and streptomycin sulfate (final concentration 100 µg/ml), and in another HIAS was replaced throughout by FCS.

The viability of monocyte-derived macrophages treated with LPS was assessed by aspirating supernatants from representative wells, staining with trypan blue and assessing cells using a haemocytometer. The remaining adherent cells were washed with PBS and then detached using trypsin. Cells were agitated to prevent clumping, stained with trypan blue, and observed using a haemocytometer.

2.2.18. Establishment of growth curves for *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* C1705.

Colonies of *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* C1705 were used to inoculate freshly autoclaved TSB. Incubation proceeded overnight in an orbital shaker at 37°C. 100µl of the resultant suspensions were added to 10ml of freshly autoclaved TSB, and incubated for 6 hours in an orbital shaker at 37°C. At the beginning of the experiment, and at each hour of the 6 hour incubation, a 100µl aliquot of the bacterial suspension was serially diluted in normal saline. From the serial dilutions, 100µl aliquots were plated out on nutrient agar and incubated overnight at 37°C. In addition, at the beginning of the experiment, 100µl aliquots of normal saline alone and TSB alone were plated out on nutrient agar and incubated overnight at 37°C, in order to exclude bacterial contamination. Colonies were counted at 24 hours, and the colony count plotted against time.

2.2.19. Assay to determine the effects of elafin on *Pseudomonas aeruginosa* and *Staphylococcus aureus* *in vitro*.

Colonies of *P. aeruginosa* PAO1 and *S. aureus* C1705 were used to inoculate freshly autoclaved TSB. Incubation proceeded overnight in an orbital shaker at 37°C. 100µl of the resultant suspensions were added to 10ml of freshly autoclaved TSB, and incubated for 3 hours (growth of each organism was known to be in logarithmic phase at this time point) in an orbital shaker at 37°C. The resulting suspensions

produced were centrifuged at 3200g for 20 minutes at room temperature, washed with 0.01M phosphate buffer (0.008M K_2HPO_4 and 0.002M KH_2PO_4) and centrifuged again under the same conditions. The pellet was resuspended in 0.01M phosphate buffer and diluted according to pre-constructed growth curves (as described in section 2.2.18) to yield an estimated bacterial concentration of 5×10^4 colony forming units (cfu)/ml.

30 μ l aliquots of each bacterial suspension were incubated with elafin peptide fragments (serially diluted in 0.01M phosphate buffer), or with human serum albumin (HSA) (serially diluted in 0.01M phosphate buffer) as a protein control, or with recombinant human SLPI (serially diluted in 0.01M phosphate buffer). The volume of each preparation of elafin, HSA or SLPI was 90 μ l, and the final concentration of elafin or HSA ranged from 1-25 μ M (in experiments using SLPI the final concentration ranged from 1-10 μ M). In each experiment, the positive control consisted of 30 μ l of bacterial suspension and 90 μ l of 0.01M phosphate buffer, while negative controls consisted of 30 μ l of 0.01M phosphate buffer and 90 μ l of elafin or HSA (each in the dose range 0-25 μ M). Incubation proceeded for 2 hours at 37°C. Serial dilutions were made in 0.01M phosphate buffer, and 100 μ l aliquots were plated onto Columbia agar. Plates were incubated for 16 hours at 37°C and colonies counted.

The number of colonies grown after incubation with elafin or SLPI in each experiment was expressed relative to the number of colonies in positive controls, and relative to the number of colonies grown upon incubation with the equimolar concentration of HSA.

2.2.20. Quantification of elafin, elastase activity, elastase inhibitory activity, myeloperoxidase, IL-8, TNF- α , and IL-1 in conditioned media *in vitro*.

Elafin concentration was established by ELISA as described in section 2.2.10. Elastase activity was estimated as described in section 2.2.12 and EIA as described in section 2.2.13. MPO activity was assessed as described in section 2.2.7. Concentrations of human IL-8, TNF- α and IL-1 were determined using commercial ELISA kits.

2.2.21. Intratracheal administration of test substances in mice.

The technique of IT administration has been described elsewhere (McLachlan et al., 2000). Mice were anaesthetised by IP injection of approximately 10µl of avertin per gramme of body weight (avertin comprised 1.25% 2,2,2-tribromoethanol and 2.5% 2-methyl-2-butanol). Mice were gently manipulated into a position of spinal extension, such that the long axis of the thorax was approximately perpendicular to the horizontal. The upper incisors were hooked over a horizontal wire strut, and the mandible pulled down by application of a light weight to the lower incisors. A bright light source was applied directly to the thoracic wall to provide transillumination of the upper mediastinum and larynx. The tongue was gently moved to one side using forceps. In this way, a clear view of the larynx and vocal cords was obtained.

The test substance was added to the plastic chamber of a blunted 25G needle (at a volume of 30-40µl depending on the experimental protocol). The blunted needle was passed between and just distal to the cords under direct vision. A 1ml syringe containing 200µl of air was gently connected to the needle, and the test substance expelled by depressing the plunger. Mice were warmed during recovery from anaesthetic.

In preliminary experiments trypan blue was administered IT in order to establish the distribution of instillate using this system.

2.2.22. Intranasal and intraperitoneal administration of test substances in mice.

Intranasal administration was performed by gently applying 30µl of test substance drop-wise around the nares of mice. In preliminary experiments trypan blue was administered IN in order to establish the distribution of instillate using this system.

Intraperitoneal administration was performed by injecting 200µl of test substance into the peritoneal cavity percutaneously.

2.2.23. Retrieval and preparation of murine bronchoalveolar lavage fluid.

BALF was prepared *ex vivo*. Mice were killed by IP injection of avertin (as described in section 2.2.21.) followed by transection of the abdominal aorta. The trachea, main bronchi and lungs were dissected from the thoracic cavity *en bloc*. Flexible nylon tubing of internal diameter 0.75mm was inserted into the trachea and secured with silk ties. 200µl or 250µl of PBS (depending on experimental protocol) was instilled immediately, and the lungs gently massaged to ensure adequate distribution and admixture. BALF was gently aspirated via the flexible nylon tubing. This process was repeated once. The total volume of BALF retrieved was measured.

BALF was centrifuged at 330g for 10 minutes at 4°C. Supernatants were immediately frozen at -80°C prior to later use. Pellets were resuspended in a known volume of PBS, and an aliquot used to count the total number of cells using a haemocytometer. A second aliquot was used to prepare cytospins, followed by staining with Diff-quick. Preparation of cytospins allowed calculation of differential cell counts.

2.2.24. Preparation of murine lungs for histological analysis.

Lungs were retrieved from mice as described in section 2.2.23. Lungs were inflated and fixed in formalin. The Department of Pathology, University of Edinburgh, kindly embedded the lungs in paraffin wax, prepared sections of 3µm thickness, and stained with haematoxylin and eosin.

2.2.25. Preparation of murine serum.

Whole blood was immediately aspirated from the peritoneal cavity after transection of the abdominal aorta, as described in section 2.2.23. Blood was centrifuged at 13,800g for 30 minutes at 4°C to prepare serum. Serum was stored at -80°C prior to use.

2.2.26. Studies of the effect of Ad-elafin on LPS-induced pulmonary neutrophilia *in vivo*.

These studies were preceded by two sets of preliminary experiments. In the first set doses of Ad-elafin ranging from 3×10^7 pfu to 1×10^9 pfu were instilled IT

(volume of each instillation 30 μ l). Mice were killed at intervals of up to 8 days post-administration. In the second set LPS was instilled IT in doses ranging from 0.1 μ g to 0.5 μ g (volume of each instillation 30 μ l). Mice were killed at 24 hours. Serum, BALF and lungs were retrieved as described in sections 2.2.23.-25.

On the basis of the preliminary experiments a protocol was devised whereby on day 0 mice received an IT instillation of Ad-elafin (3×10^7 pfu in PBS), Ad-lacZ (3×10^7 pfu in PBS) or PBS (volume of each instillation 30 μ l); on day 5 mice from each group received an instillation of either PBS or 0.5 μ g LPS (volume of each instillation 30 μ l). The mice were killed on day 6 and BALF, lungs and serum were retrieved as described in sections 2.2.23.-25.

2.2.27. Staining of β -galactosidase in Ad-lacZ transfected murine lungs.

Mice received an IT instillation of Ad-lacZ (3×10^7 pfu in PBS, final volume 30 μ l) followed 5 days later by an IT instillation of LPS (either 0.5 μ g or 5 μ g, final volume 30 μ l). The mice were killed 24 hours later and lungs retrieved as described in section 2.2.23. 250 μ l of fixative (PBS containing 2% formaldehyde and 0.2% glutaraldehyde) was instilled, and the end of the trachea tied off with a silk suture. The lungs were immersed in a tube containing 2% formaldehyde and 0.2% glutaraldehyde in PBS, and incubated at 4°C for 1 hour. The surface of the lungs was washed with PBS and fixative was poured out of the lung. 250 μ l of PBS was instilled IT and discarded; this process was repeated once. 250 μ l of staining solution (as described in section 2.2.4) was then instilled, the trachea was tied off with a silk suture, and the lungs incubated overnight at 37°C. The Department of Pathology, University of Edinburgh kindly photographed the lungs, which they then embedded in paraffin wax, sectioned, and stained with nuclear fast red.

2.2.28. Immunohistochemical localisation of human elafin in murine lungs.

Mice were treated IT with Ad-elafin (3×10^7 pfu), Ad-lacZ (3×10^7 pfu), or PBS on day 0, and with PBS or 0.5 μ g LPS on day 5, then were killed on day 6 as described in section 2.2.23. Blocks of paraffin-embedded lung were prepared as in section 2.2.24. Sections 3 μ m thick were prepared and mounted on glass slides.

Slides were incubated in xylene for 45 minutes at room temperature, then in 99.8% ethanol for 3 minutes at room temperature. The slides were washed with tap

water followed by incubation for 30 minutes at room temperature in methanol containing 3% H₂O₂. The slides were rinsed sequentially in water and Tris buffered saline (TBS), then exposed to decreasing concentrations of ethanol (90%, 70% and 50% respectively, each for 30 seconds). Non-specific binding sites were blocked by incubating with 20% normal goat serum (NGS) in TBS (total volume 100µl per slide) for 20 minutes at room temperature. 100µl of primary antibody was applied overnight at room temperature and comprised rabbit anti-human elafin IgG diluted 1000-fold into 20% NGS in TBS. Two controls were used, namely TBS alone and pre-immune rabbit serum diluted 1000-fold into 20% NGS in TBS (in each case 100µl was applied at room temperature overnight).

Slides were washed with TBS, then treated for 30 minutes at room temperature with goat anti-rabbit immunoglobulin diluted 300-fold into 20% NGS in TBS. Further washes in TBS were performed prior to addition of avidin-biotin-horseradish peroxidase (HRP) in avidin-biotin buffer for 30 minutes at room temperature. Slides were again washed in TBS prior to addition of acetate buffer, pH5.0 for 5 minutes at room temperature.

Substrate (DAB containing 1.5% H₂O₂) was added for 10 minutes at room temperature, prior to washing with water. Slides were then treated sequentially with haematoxylin and Scott's Tap Water Solution before being washed with water. Finally, slides were exposed to increasing concentrations of ethanol (50%, 70% and 90% respectively), and mounted using standard mounting fluid.

2.2.29. Preparation of *Pseudomonas aeruginosa* PAO1 for administration *in vivo*.

Pseudomonas aeruginosa PAO1 was inoculated into nutrient broth containing 0.5% yeast extract, and incubated overnight at 37°C with continuous orbital shaking. The bacterial suspension was centrifuged at 3200g for 15 minutes at room temperature, and the pellet washed in 0.01M phosphate buffer, pH7.0. The pellet was resuspended in 0.01M phosphate buffer and the absorbance at 590nm measured. The absorbance value was adjusted to between 1.40 and 1.45 by dilution with 0.01M phosphate buffer (in some preliminary experiments further dilutions were made). 34-40µl aliquots of the final suspension (depending on the experimental protocol) were used immediately for IT instillation. Simultaneously, an aliquot was serially diluted, plated onto PIA, and incubated at 37°C overnight, so that the viable bacteria in the starting instillate could be quantified.

2.2.30. Preparation of biological fluids and organs after IT delivery of *Pseudomonas aeruginosa* PAO1 to mice.

BALF was prepared as described in section 2.2.23., with the exception that a 50µl aliquot of whole BALF was serially diluted and applied to PIA. Colonies were counted after overnight incubation at 37°C.

Whole blood was retrieved as described in section 2.2.25. and 100µl was applied immediately to PIA. Colonies were counted after overnight incubation at 37°C. Whole blood which was not used to assess bacterial counts was used to prepare serum as described in 2.2.25.

The spleen was dissected free, placed in 1ml of 0.01M phosphate buffer and homogenised for 30 seconds at room temperature using an Omni hand held homogeniser (Camlab, Cambridge, UK). An aliquot of 100µl was applied to PIA. Colonies were counted after overnight incubation at 37°C.

The right lung was dissected free, placed in 1ml of 0.01M phosphate buffer and homogenised for 1 minute at room temperature. The homogenate was serially diluted in 0.01M phosphate buffer. 100µl aliquots were applied to PIA. Colonies were counted after overnight incubation at 37°C.

The left lung was dissected free, weighed, and immediately snap frozen in liquid nitrogen. Upon thawing lungs were immediately placed in homogenisation buffer, consisting of 100mM sodium acetate, 20mM EDTA, 1% hexadecyl trimethyl ammonium bromide (after LeVine et al., 1998). Homogenisation proceeded for 1 minute at room temperature. The homogenate was then centrifuged at 8200g for 30 minutes at room temperature. The supernatant was aspirated and immediately used to determine the concentration of MPO as described in section 2.2.8.

In each experimental protocol involving IT instillation of *Pseudomonas aeruginosa* PAO1, a number of representative mice did not have lungs removed for homogenisation. Instead, lungs were fixed and used for histological analysis as described in section 2.2.24.

2.2.31. Studies of the effect of Ad-elafin on lung injury induced by *Pseudomonas aeruginosa*.

Preliminary experiments were performed in which varying doses of *Pseudomonas aeruginosa* PAO1 were prepared and administered IT as described in sections 2.2.21. and 2.2.29. Mice were killed at either 4 hours or 24 hours. The aim of these experiments was to determine a dose of *Pseudomonas aeruginosa* PAO1 which would consistently produce sublethal lung injury.

On the basis of the preliminary experiments, the following protocol was designed. On day 0, mice received an IT instillation of Ad-elafin (3×10^7 pfu in PBS), Ad-lacZ (3×10^7 pfu in PBS) or PBS (volume of each instillation 40 μ l); on day 5 mice from each group received an IT instillation of *Pseudomonas aeruginosa* PAO1 (34 μ l of a suspension with an absorbance value of between 1.40 and 1.44 at 590nm). Mice were killed on day 6. BALF, serum, spleen and lungs were retrieved as described in section 2.2.30.

2.2.32. Quantification of elafin, EIA, protein, albumin, MPO, MIP-2, TNF- α , murine keratinocyte-derived chemokine (mKC), monocyte chemotactic protein 1 (MCP-1) and MIP-1 α in murine BALF.

Elafin concentrations were quantified by ELISA as described in section 2.2.10. EIA was estimated as described in section 2.2.13. Protein concentration was measured using a kit based on the bicinchoninic acid method, using albumin as standard. Murine albumin concentrations were measured using a commercial ELISA kit. MPO activity was measured by adding 50 μ l of BALF directly to 50 μ l of MPO substrate (0.1 mg/ml TMB, 0.03% H₂O₂, in 0.1M Na acetate-citrate, pH 4.9) and quantifying the change in absorbance at 630nm, as described in section 2.2.8. MIP-2 and TNF- α concentrations were established using commercial ELISA kits. MIP-1 α , mKC and MCP-1 ELISAs were kindly performed by Professor T. Standiford, University of Michigan, Ann Arbor, MI, USA.

2.2.33. Statistical analysis.

When numerical variables were normally distributed, Student's t-test was used. When numerical variables did not conform to a normal distribution, non-parametric statistical analysis was performed. Three group comparisons were

performed using the Kruskal-Wallis test. Paired comparisons were analysed using Wilcoxon's signed rank test, unpaired comparisons using the Mann-Whitney U-test, and correlations using Spearman's rank correlation test. Nominal data were analysed using the chi-squared test.

Statistical significance was assigned to a p value of < 0.05 .

CHAPTER 3

THE EFFECT OF ELAFIN GENE AUGMENTATION ON HNE- AND NEUTROPHIL-MEDIATED CELL DAMAGE *IN VITRO*

3.1. AIMS

The experiments described in this chapter were performed with the aim of answering three fundamental questions.

Firstly, is the Ad-elafin vector capable of efficiently transfecting pulmonary epithelial cells with subsequent expression of functional human elafin at a level higher than that produced constitutively?

If so, is genetic augmentation of human elafin sufficient to protect pulmonary epithelial cells against the damaging effects of purified HNE?

If so, is genetic augmentation of human elafin capable of protecting pulmonary epithelial cells against the damaging effects of whole, activated human neutrophils?

3.2. RESULTS

3.2.1. Adenoviral transfection of A549 cells.

Transfection of A549 cells with recombinant adenovirus (Ad-lacZ) was readily achieved, with transfection efficiency approaching 100% at an moi of 50 pfu (Figure 1). However evidence was still required for expression of functional human elafin after Ad-elafin transfection. Elafin was secreted from A549 cells in a dose-dependent manner after treatment with Ad-elafin (Figure 2A). There was no associated increase in elafin secretion upon transfection with Ad-lacZ, indicating that viral transfection *per se* was not responsible for the secretion stimulated by Ad-elafin (Figure 2A). Furthermore Ad-elafin transfection resulted in a corresponding dose-dependent inhibition of purified HNE (Figure 2B). Once again, transfection with Ad-lacZ did not stimulate elastase inhibition, thus effectively excluding the possibility that viral infection may have driven significant release of elastase

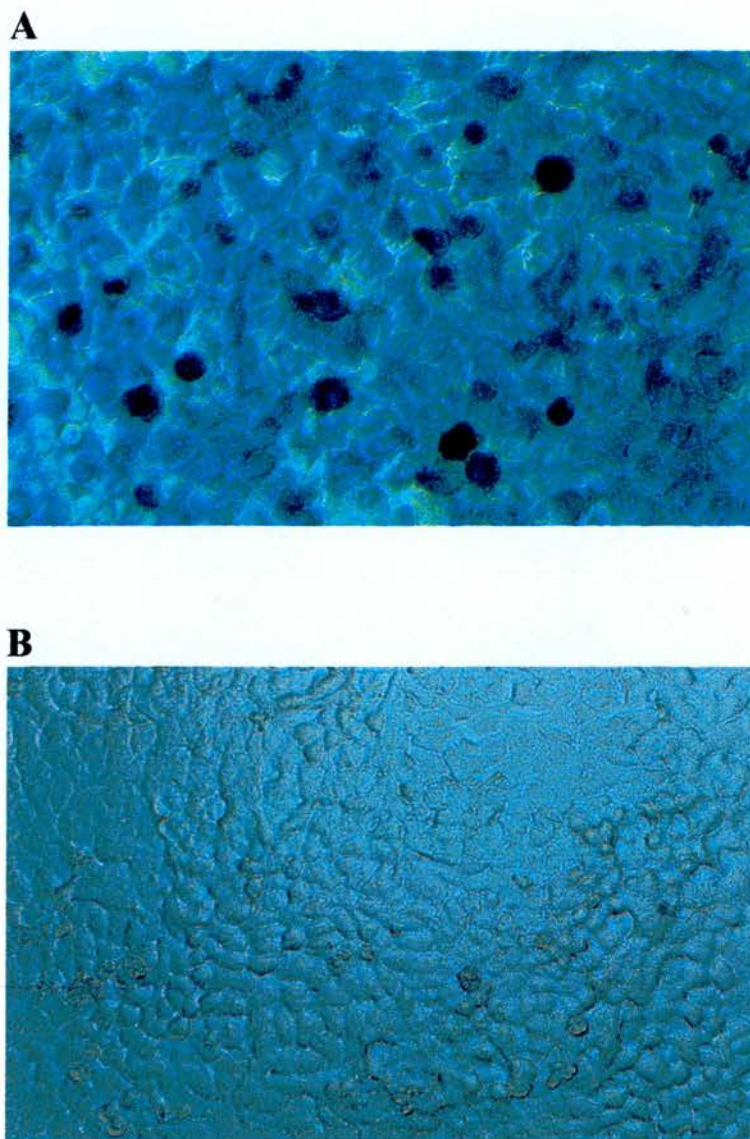


Figure 1. Adenovirus efficiently transfects A549 cells.

A549 cells were treated with Ad-lacZ (PANEL A; moi of 50 pfu - original magnification x 200) or vehicle alone (PANEL B - original magnification x 150) and incubated in serum-free DMEM for 24 hours. Cells were then treated with X-gal stain. Blue colouration indicates expression of the β -galactosidase gene.

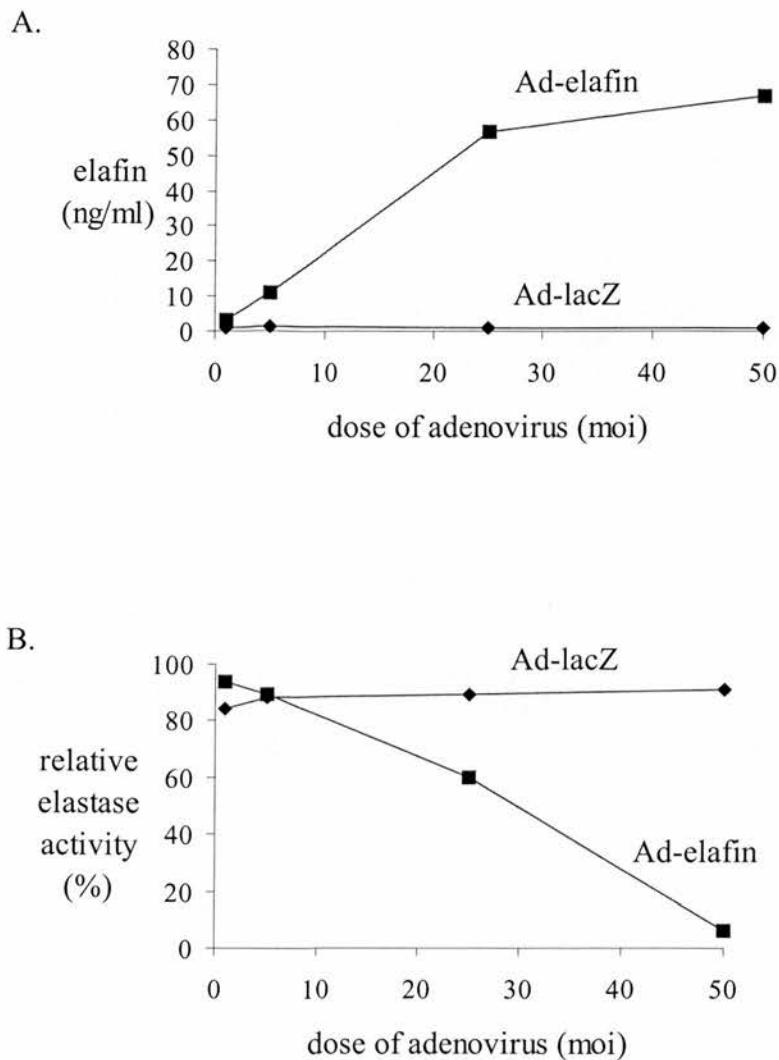


Figure 2. Functional elafin is secreted by A549 cells transfected with Ad-elafin.

A549 cells were transfected with Ad-elafin or Ad-lacZ (dose range 1-50 moi) or vehicle alone, then incubated in serum-free DMEM for 48 hours. HNE (final concentration 4 μ g/ml) was added for 16 hours and supernatants retrieved. Elafin concentration (PANEL A) and elastase activity (PANEL B) were measured. Elastase activity is expressed relative to that in supernatants retrieved from untransfected cells.

Results are derived from a single representative experiment.

inhibitors after Ad-elafin treatment (Figure 2B). These data suggested that Ad-elafin transfection was capable of generating high concentrations of functionally active human elafin from A549 cells.

Ad-elafin transfection was not associated with overt morphological damage to the cellular monolayer (as evidenced by light microscopy). This impression was corroborated when A549 cell monolayers treated with Ad-elafin or Ad-lacZ (each at moi of 50 pfu) and incubated in serum-free DMEM for 48 hours were detached using trypsin, washed, and exposed to trypan blue. The percentage of non-viable cells (ie those staining positively for trypan blue) was 14% among untransfected cells, 9% among Ad-lacZ treated cells, and 12% among Ad-elafin treated cells. However viral transfection was associated with a higher rate of extrusion of cells from A549 cell monolayers; 6,100 untransfected cells, 12,200 Ad-lacZ treated cells, and 16,000 Ad-elafin treated cells were recovered from supernatants after incubation in serum-free medium for 3 days (all values are means of 3 separate experiments, no significant differences between groups; confluent monolayers typically contain approximately 300,000 cells). In none of these experiments were deficiencies in the monolayer found, suggesting that cells extruded were replaced (or at least that the corresponding surface area was covered).

Given that transfection with Ad-elafin at an moi of 50 generated marked elastase inhibition without morphological damage to the epithelial monolayer, elafin production at this dose was characterised further. Although the concentration of elafin produced by Ad-elafin transfection was variable it was in general approximately 100 times that from either untransfected or Ad-lacZ treated cells (Figure 3).

Elafin was secreted from untransfected (and Ad-lacZ treated) A549 cells in all experiments performed (Figure 3). Constitutive elastase inhibition by A549 cells was simply demonstrated by adding known concentrations of HNE to serum-free DMEM which had either been incubated with A549 cells at 37°C for 24 hours, or incubated in wells containing no cells at 37°C for 24 hours (Figure 4A). Elastase activity was lower in the conditioned medium, thereby suggesting generation of elastase inhibitors by A549 cells (Figure 4A). While the experiment described in Figure 2 suggested that elafin augmentation accounts for the increase in anti-elastase activity after Ad-elafin transfection, the demonstration of constitutive elastase inhibition in untransfected

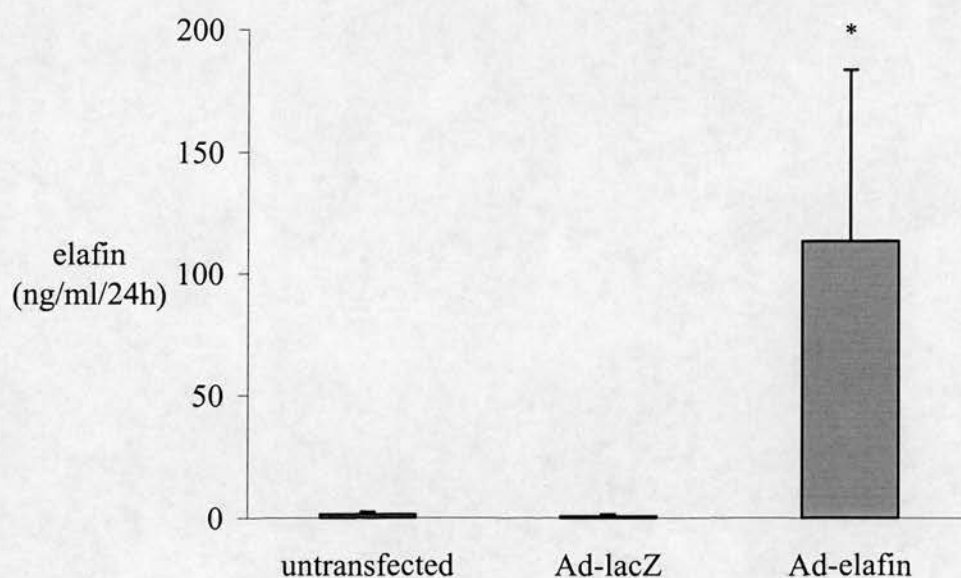


Figure 3. Ad-elafin transfection results in augmentation of elafin secretion from A549 cells.

A549 cells were treated with Ad-elafin or Ad-lacZ (moi 50 in each case) or vehicle alone, and incubated in serum-free medium for 48-72 hours. Supernatants were retrieved and elafin concentration measured. Data are presented as means and standard deviations (n=4).

*= Significant difference, $p < 0.05$, when compared to each of the other treatments.

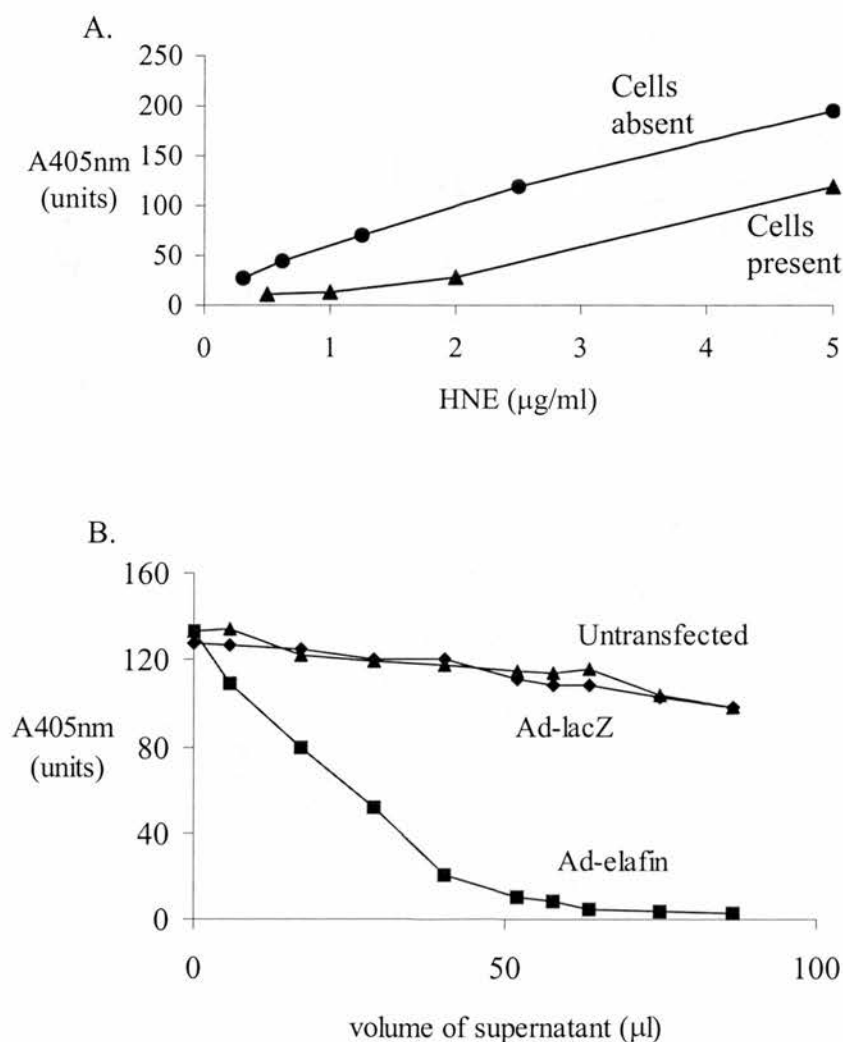


Figure 4. A549 cells have constitutive elastase inhibitory capacity which is augmented by transfection with Ad-elafin.

PANEL A – Increasing concentrations of HNE were added to serum-free DMEM which had been incubated with untransfected A549 cells ('Cells present'), or in wells containing no cells ('Cells absent') for 24 hours. Elastase activity was measured as a function of time, and is represented as absorbance at 405nm (A405nm) after the addition of elastase specific substrate. Results are derived from a single representative experiment.

PANEL B – A549 cells were treated with Ad-elafin or Ad-lacZ (each at 50 moi) or vehicle alone and incubated in serum-free DMEM for 48 hours. Increasing volumes of supernatant were incubated with HNE (22.5ng in 10 μl) for 30 minutes. Elastase activity was measure as described for PANEL A.

cells stimulated the need to characterise the relative contribution of elafin to total EIA in A549 cells, whether they be untransfected or treated with adenovirus.

Transfected (moi of 50 for Ad-lacZ and Ad-elafin) and untransfected A549 cells were therefore incubated in serum-free DMEM for 48 hours and supernatant retrieved. The concentration of elafin in conditioned medium was 2.3 ng/ml from untransfected cells, 0.3 ng/ml from Ad-lacZ treated cells, and 180 ng/ml from Ad-elafin treated cells. For each condition increasing volumes of supernatant were incubated with a known concentration of HNE (22.5 ng in 10 μ l) and the resulting elastase activity measured, leading to the generation of the curves described in Figure 4B. The HNE used in this experiment was known to be approximately 50% active. Extrapolation of the curves to the abscissa allows an estimate of the minimum quantity of medium required to inhibit completely the known concentration of HNE, assuming 1:1 stoichiometric binding of elastase inhibitors with HNE and 50% activity of HNE (after Bieth, 1980). Under these conditions estimated EIAs of conditioned medium were calculated at 0.8 nM for untransfected cells, 0.75 nM for Ad-lacZ treated cells and 9.1 nM for Ad-elafin treated cells.

Although a number of variables influence these calculations, some limited conclusions can be drawn. Firstly, given the known concentration of elafin antigen in conditioned media, then even if elafin were fully active it would contribute no more than 29% to the total EIA of untransfected cells, and no more than 4% to the EIA of Ad-lacZ treated cells. Therefore it can be confidently stated that the majority of EIA constitutively produced by A549 cells is not attributable to elafin. Furthermore one can confidently conclude that adenoviral transfection (with Ad-lacZ) does not up-regulate EIA. On the other hand Ad-elafin markedly up-regulated EIA in A549 cells.

The data described in this section suggested that adenoviral augmentation of elafin may be capable of inhibiting HNE-mediated damage of A549 cells.

3.2.2. The effect of Ad-elafin transfection on HNE-mediated damage of A549 cells.

The application of purified HNE resulted in a characteristic pattern of damage to untransfected A549 cells incubated in serum-free medium. In general final concentrations of HNE of approximately 1 μ g/ml resulted in cells taking on a more spindle shaped morphology, with gaps beginning to appear between cells. At 2 μ g/ml gaps between cells were more pronounced, some cells were seen to detach

from the monolayer, and those remaining in contact with the plate were rounded and granular. At 4 $\mu\text{g/ml}$ cellular detachment was invariably present, and the few cells remaining in contact with plates were rounded and granular. At 4 $\mu\text{g/ml}$ of HNE cells often detached in sheets (ie within the sheet cell contacts appeared to be preserved), although individual cells were also identified in supernatants. Because HNE at 4 $\mu\text{g/ml}$ was invariably associated with severe cellular damage and detachment, this concentration was used in most experiments designed to assess whether Ad-elafin could ameliorate HNE-mediated damage.

In a preliminary experiment it was demonstrated that Ad-elafin transfection (moi ranging from 10-50) could markedly reduce the number of cells released from A549 monolayers after application of HNE at 4 $\mu\text{g/ml}$ for 24 hours (Figure 5A). This protective effect was associated with inhibition of residual elastase activity which was almost completely abolished when Ad-elafin was applied at an moi of 50 (Figure 5B). Ad-lacZ treatment was also associated with a reduction in detachment and with a modest inhibition of elastase activity, the protective effect being less pronounced than that observed after Ad-elafin treatment (Figure 5).

A time course study demonstrated that HNE-mediated detachment of Ad-lacZ treated cells began between 12 and 24 hours after application of the enzyme (Figure 6A). Prior treatment of A549 cells with Ad-elafin conferred marked protection against cellular detachment (Figure 6A). However, detachment of Ad-elafin treated A549 cells did occur, despite the fact that an moi of 50 was associated with almost complete inhibition of HNE throughout the 24 hour period (Figure 6B).

The preliminary experiments described stimulated more comprehensive studies of the effects of Ad-elafin (moi of 50) in protecting epithelial cells against HNE. Application of HNE at 2 $\mu\text{g/ml}$ to Ad-lacZ treated cells (or untransfected cells) consistently resulted in disruption of the epithelial monolayer (Figure 7, Panel B), with rounding and spindling of cells most pronounced around areas of denudation. The destructive process continued in a dose-dependent manner, such that HNE at 4 $\mu\text{g/ml}$ resulted in almost complete separation of cells from their plastic base, the remaining cells being small and rounded (Figure 7, Panel C). In contrast pre-treatment with Ad-elafin resulted in considerable protection against the effects of HNE at 4 $\mu\text{g/ml}$, there being relatively minor disruption of the epithelium (Figure 7, Panel D).

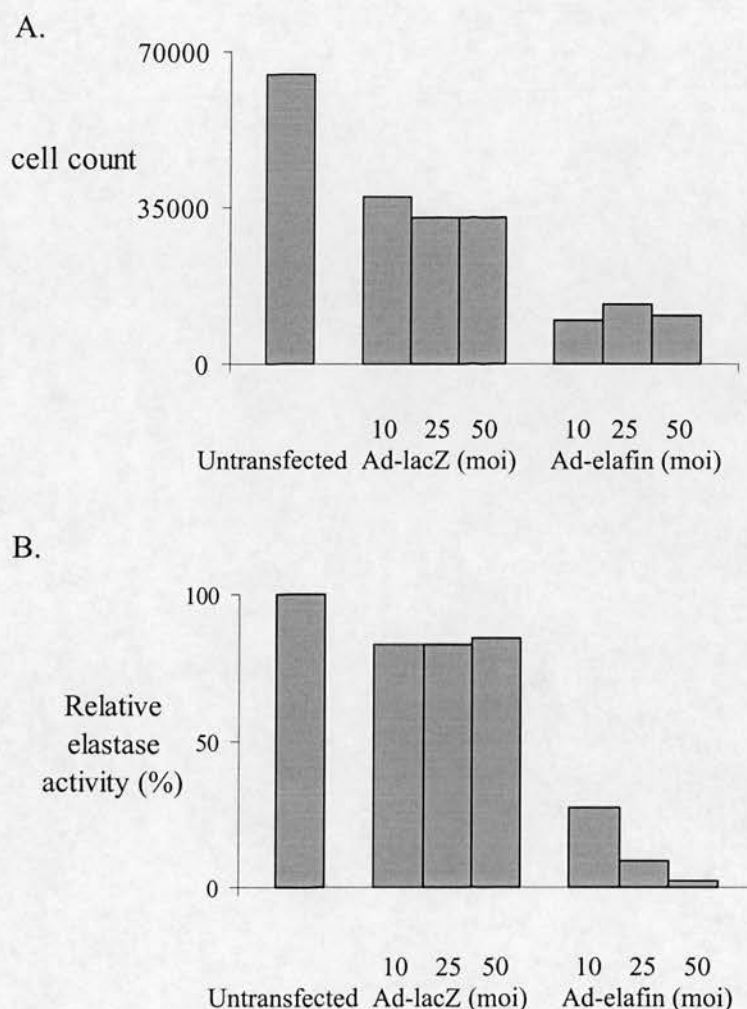


Figure 5. Ad-elafin transfection of A549 cells is associated with a reduction in HNE-mediated cell detachment and elastase activity.

A549 cells were treated with Ad-elafin or Ad-lacZ (dose range 10-50 moi) or vehicle alone, and incubated in serum-free medium for 72 hours prior to the addition of HNE (final concentration 4 μ g/ml) for 24 hours. Supernatants were retrieved for measurement of cell count (PANEL A) and residual elastase activity (PANEL B; data expressed relative to the elastase activity in supernatants from untransfected cells). Data are derived from a single experiment.

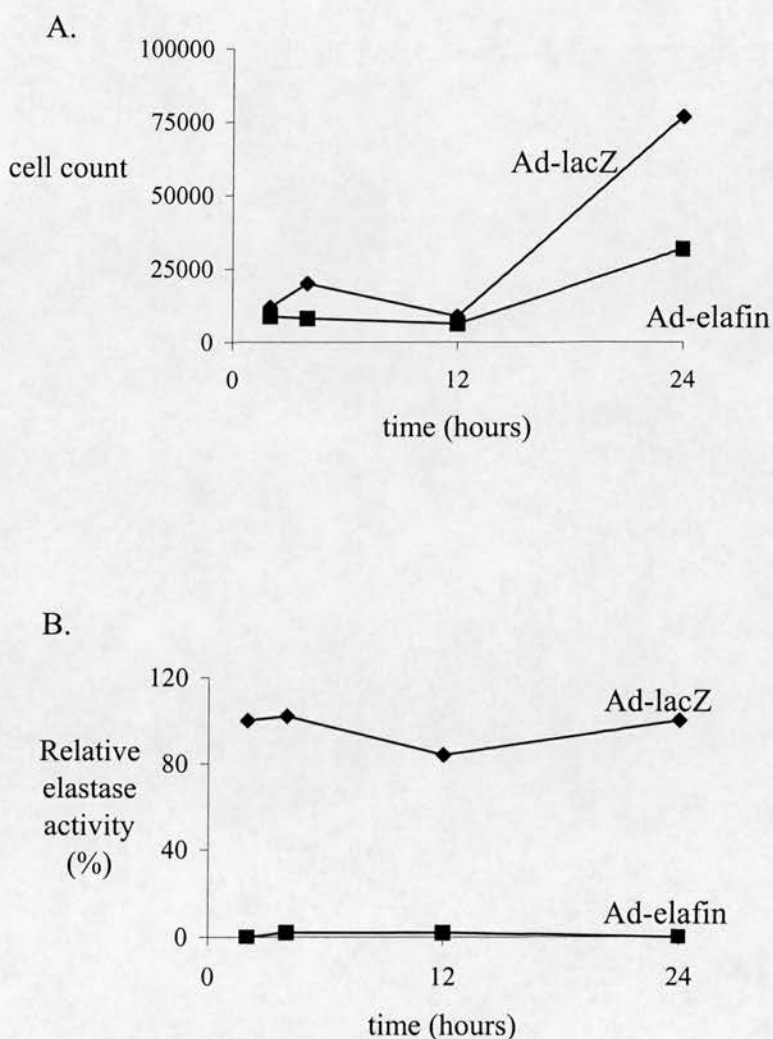


Figure 6. HNE-mediated detachment of A549 cells occurs after 12 hours.

A549 cells were treated with Ad-elafin or Ad-lacZ (50 moi in each case) and incubated in serum-free medium for 3 days prior to the addition of HNE (final concentration 4 μ g/ml) for 24 hours. Cell count (PANEL A) and residual elastase activity (relative to that in supernatants from Ad-lacZ cells at 2 hours) were measured in the retrieved supernatants.

Data are derived from a single experiment.

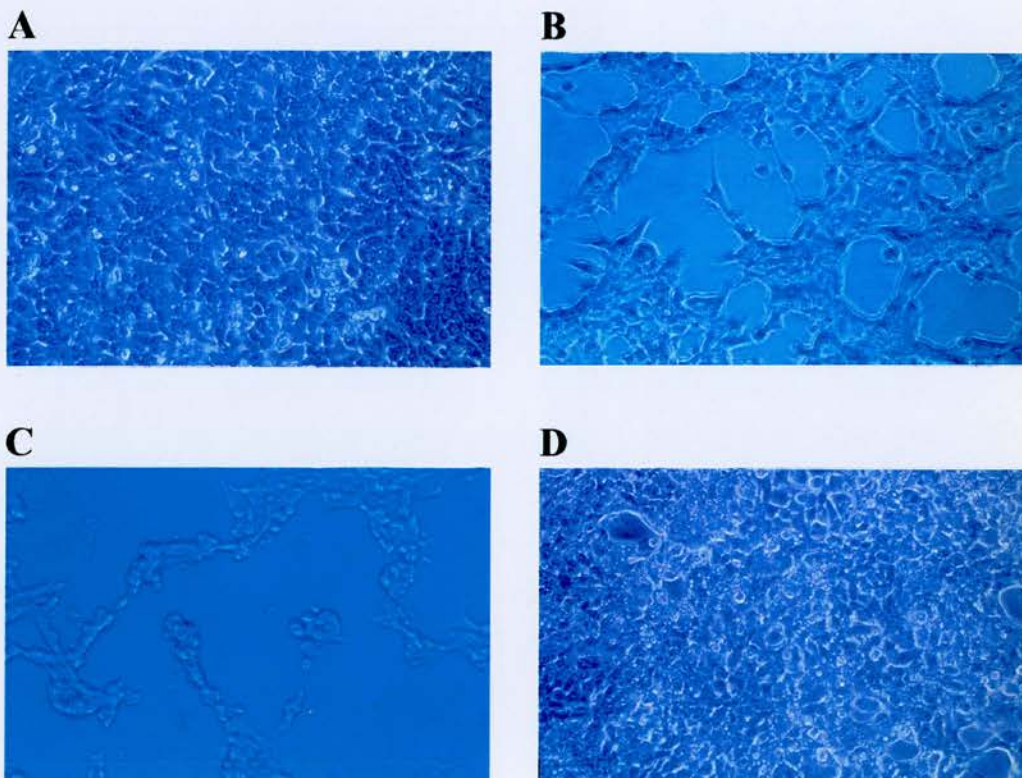


Figure 7. Transfection of A549 cells with Ad-elafin confers protection against HNE-mediated cell damage.

A549 cells were treated with Ad-elafin or Ad-lacZ (moi 50 in each case) or vehicle alone and incubated in serum-free medium for 2 days prior to the addition of HNE (final concentration ranging from 2-4 μ g/ml) for 16 hours.

PANEL A - appearance of untransfected cells to which no HNE was added.

PANEL B - appearance of Ad-lacZ treated cells exposed to HNE at 2 μ g/ml.

PANEL C - appearance of Ad-lacZ treated cells exposed to HNE at 4 μ g/ml.

PANEL D - appearance of Ad-elafin treated cells exposed to HNE at 4 μ g/ml (original magnification x 100 in each case).

The morphological appearance of untransfected cells resembled that of Ad-lacZ treated cells at all doses of HNE. The blue colour was conferred by a photographic filter used to enhance contrast.

The protective effect was quantified by determining the number of epithelial cells liberated into supernatants after application of HNE at 4 $\mu\text{g/ml}$ (Figure 8). Ad-elafin transfection was associated with a dose-dependent reduction in cell detachment, in keeping with the appearances in Figure 7. Interestingly there was considerable variation in the degree of protection conferred by Ad-elafin at an moi of 10, but very consistent protection with an moi of 50. This proved to be consistent with morphological findings, in that protection was invariably conferred by an moi of 50 (Figure 7) but was not universally observed at an moi of 10. A further point to make is that Ad-lacZ transfection resulted in a 23% reduction in detachment as compared with untransfected cells (Figure 8), but this did not reach statistical significance.

Pursuing the theme of HNE-mediated damage of A549 cells further, a separate experiment was performed in which Ad-elafin and Ad-lacZ transfected cells (moi of 50 in each case) were incubated in serum-free medium for 24 hours prior to addition of HNE (4 $\mu\text{g/ml}$). Sixteen hours later, the number of cells in supernatants tended towards the pattern described in Figure 8 (ie release of 45000 untransfected cells, 45000 Ad-lacZ treated cells, and 3750 Ad-elafin treated cells). The cells remaining attached to the plastic base were incubated with trypsin until full detachment was established, at which point 400 μl of serum containing medium was immediately added to neutralise trypsin. The cells were spun and resuspended for counting. Surprisingly, no untransfected cells or Ad-lacZ treated cells were identified at light microscopy, despite there being complete denudation of cells from the plastic plates. In contrast, the expected number of Ad-elafin cells were retrieved (ie 97.7% of the count retrieved from Ad-elafin treated cells to which HNE had not been applied). These data imply that HNE had lysed the untransfected/Ad-lacZ treated cells and/or that a proportion of significantly damaged cells had been rendered susceptible to lysis by trypsin. Importantly, 95% of the Ad-elafin treated cells exposed to HNE remained viable (as compared to 86%, 91% and 88% respectively in untransfected, Ad-lacZ treated and Ad-elafin treated cells to which no HNE was added). In summary, Ad-elafin treatment preserves both adhesion and viability of an epithelial cell monolayer exposed to HNE.

Further quantitative evidence for a protective effect of Ad-elafin was provided by studying radiolabelled A549 cells (Figure 9). In keeping with the patterns described in Figures 7 and 8, Ad-elafin transfection resulted in lower levels of released radiolabel.

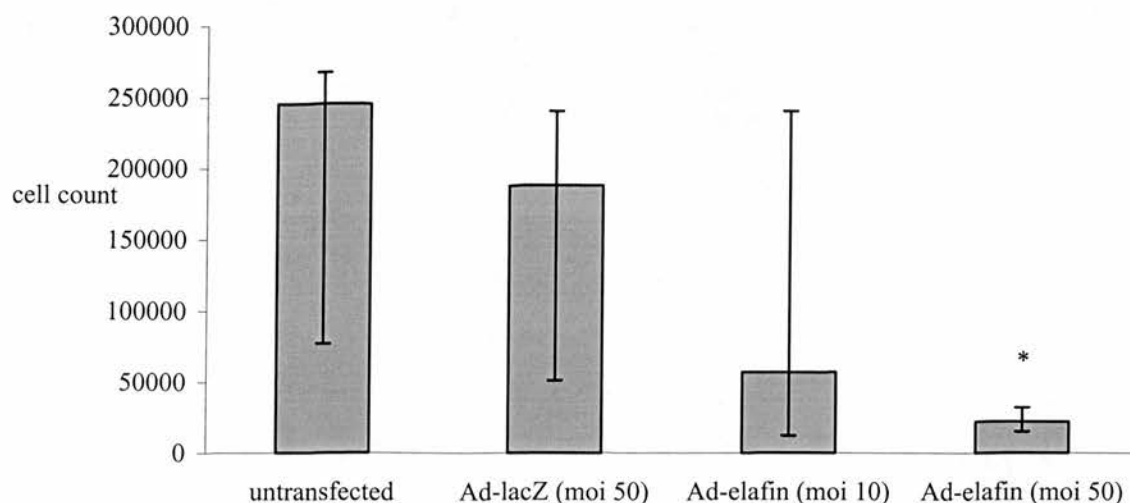


Figure 8. Transfection of A549 cells with Ad-elafin results in a dose-dependent inhibition of HNE-mediated cell detachment.

A549 cells were treated with Ad-elafin (moi 10 or 50) or Ad-lacZ (moi 50) or vehicle alone, and incubated in serum-free medium for 48-72 hours prior to the addition of HNE (final concentration 4 μ g/ml). The number of cells in retrieved supernatants was estimated.

Data are presented as medians and interquartile ranges (n=7, except in the case of Ad-elafin at 10 moi, where n=4). * = Significant difference, $p < 0.05$, when compared to untransfected cells or Ad-lacZ treated cells.

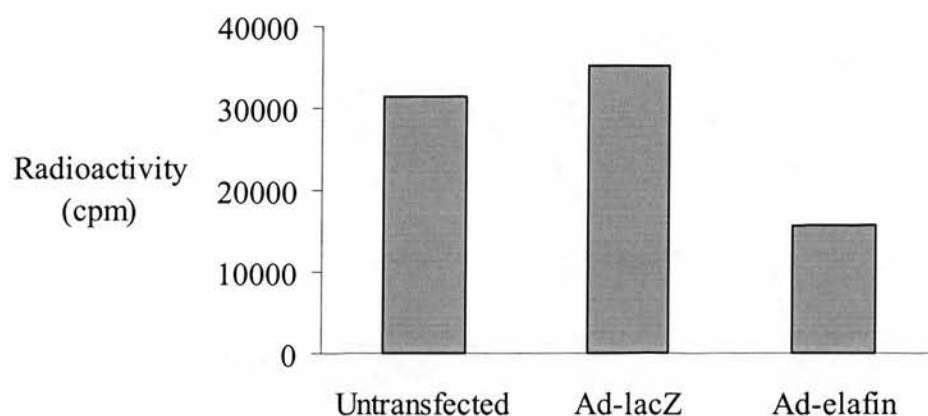


Figure 9. Ad-elafin transfection reduces release of 111 indium from radiolabelled A549 cells.

Radiolabelled A549 cells were treated with Ad-elafin or Ad-lacZ (moi of 50 for each) or vehicle alone, and incubated in serum-free medium for 24 hours prior to the addition of HNE (final concentration 4 μ g/ml) for a further 16 hours. Radioactive label in retrieved supernatants was quantified. Results are derived from a single experiment.

Interestingly the addition of HNE to Ad-elafin treated cells was associated with a reduction in the concentration of elafin detectable by ELISA (Figure 10, Panel A) or dot-blot assay (Figure 10, Panel B). A similar reduction in detectable elafin was observed after addition of HNE to untransfected and Ad-lacZ treated cells (Figure 10). The dot-blot assay reinforces two points made previously. Firstly Ad-elafin transfection results in a dose-dependent increase in elafin secretion. Secondly, there is further evidence to support the trend for a small reduction in elafin secretion from Ad-lacZ treated cells as compared with untransfected cells.

The reduction in detectable elafin antigen after addition of HNE (Figure 10) was studied further in the absence of A549 cells. Recombinant elafin (at a concentration of 10 ng/ml) was incubated with increasing concentrations of HNE to give molar ratios (HNE:elafin) of 1:2 and 2:1. The elafin concentration was measured by ELISA. When no HNE was added, 10.3 ng/ml was detected. At a molar ratio of 1:2 5.9 ng/ml was detected, and at a ratio of 2:1 4.3 ng/ml was detected. It seems likely therefore that complexed elafin is less easily detected by anti-elafin antibody than is "free" elafin, and/or that elafin is cleaved by HNE yielding undetectable fragments.

The protection conferred against HNE by transfection of A549 cells with Ad-elafin (Figures 7,8 and 9) was associated with a significant inhibition of HNE activity (Figure 11). The inhibition was dose-dependent, with almost complete inhibition of HNE at an moi of 50. Ad-lacZ treatment did not result in significant elastase inhibition as compared with that observed in untransfected cells.

The protective effect of Ad-elafin was examined further by assessing the influence of anti-elafin antibody. Preliminary experiments established a concentration of anti-elafin antibody (80 µg/ml) which consistently neutralised the inhibitory effects of 800 ng/ml of elafin. The experiments described in Figures 8 and 11 were therefore repeated, with the exception that an excess of anti-elafin antibody (or control antibody) was incubated with supernatants for 1 hour prior to the addition of HNE (Figure 12). The characteristic reduction in cell detachment associated with Ad-elafin transfection was again observed (Figure 12, Panel A) and control antibody had no influence on this process. However anti-elafin antibody resulted in a partial restoration of HNE-induced cell detachment. In accordance with this finding, anti-elafin antibody also conferred partial restoration of morphological cell damage. Furthermore, the characteristic reduction in elastase activity associated with Ad-

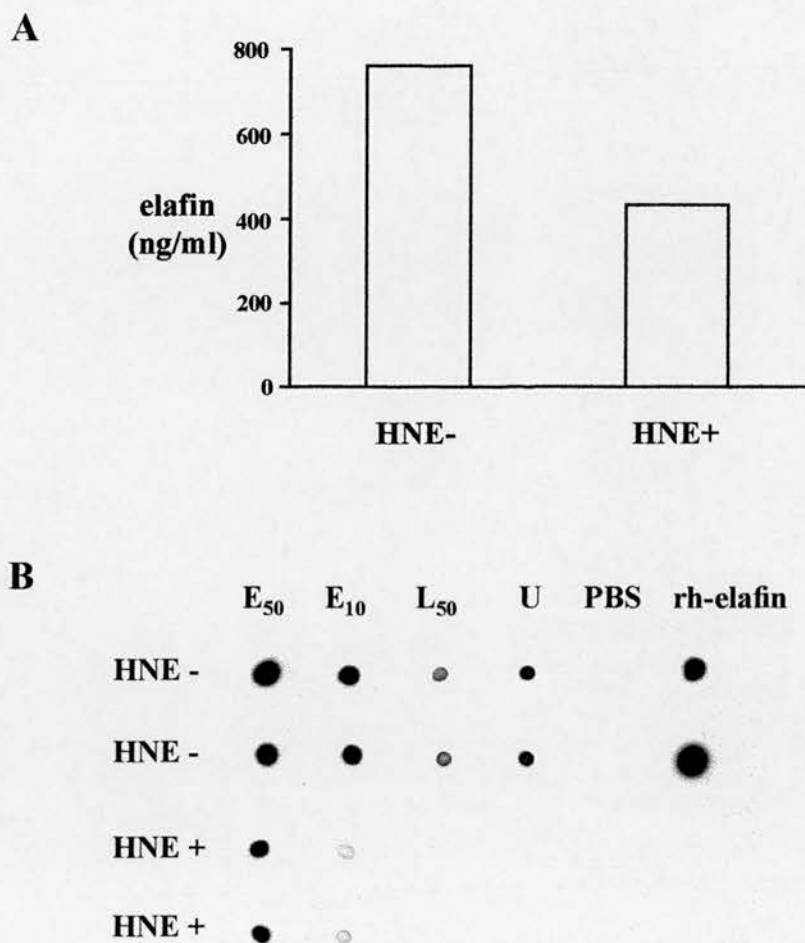


Figure 10. The presence of HNE leads to reduced concentrations of detectable elafin antigen.

A549 cells were treated with Ad-elafin or Ad-lacZ (moi of 10-50 for each) or vehicle alone and incubated in serum-free medium for 72 hours prior to the addition of HNE (final concentration 4µg/ml) or vehicle alone for a further 16 hours.

Panel A illustrates elafin concentration (measured by ELISA) in supernatants from Ad-elafin treated cells (moi of 50) receiving vehicle (HNE-) or HNE (HNE+).

Panel B represents a dot-blot assay; E₅₀ = Ad-elafin (moi 50), E₁₀ = Ad-elafin (moi 10), L₅₀ = Ad-lacZ (moi 50), U = untransfected; HNE- = vehicle alone added to cells, HNE+ = HNE added to cells; PBS = PBS negative control; rh-elafin = recombinant human elafin (as a positive control; upper panel 0.1µg/ml, lower panel 1µg/ml).

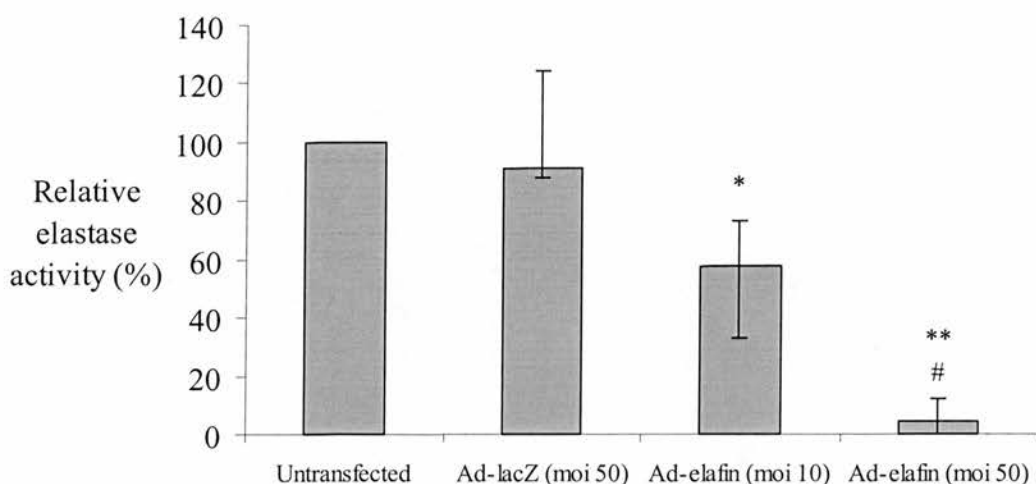


Figure 11. Transfection of A549 cells with Ad-elafin results in a dose-dependent inhibition of elastase activity.

A549 cells were treated with Ad-elafin (moi 10 or 50) or Ad-lacZ (moi 50) or vehicle alone, and incubated in serum-free medium for 48-72 hours prior to the addition of HNE (final concentration 4 μ g/ml). Residual elastase activity was measured in supernatants after 16 hours of exposure to HNE.

Elastase activity was expressed relative to the elastase activity in supernatants from untransfected cells. Data are presented as medians and interquartile ranges (n=11, except in the case of Ad-elafin at 10 moi, where n=7); The “Untransfected” column has no error bars because the value obtained was regarded as unity in all experiments. * = Significant difference, $p < 0.05$, comparing Ad-elafin (moi 10) with untransfected cells and Ad-lacZ. ** = Significant difference, $p < 0.005$, comparing Ad-elafin (moi 50) with untransfected cells and Ad-lacZ. # = Significant difference, $p < 0.05$, comparing Ad-elafin (moi 50) with Ad-elafin (moi 10).

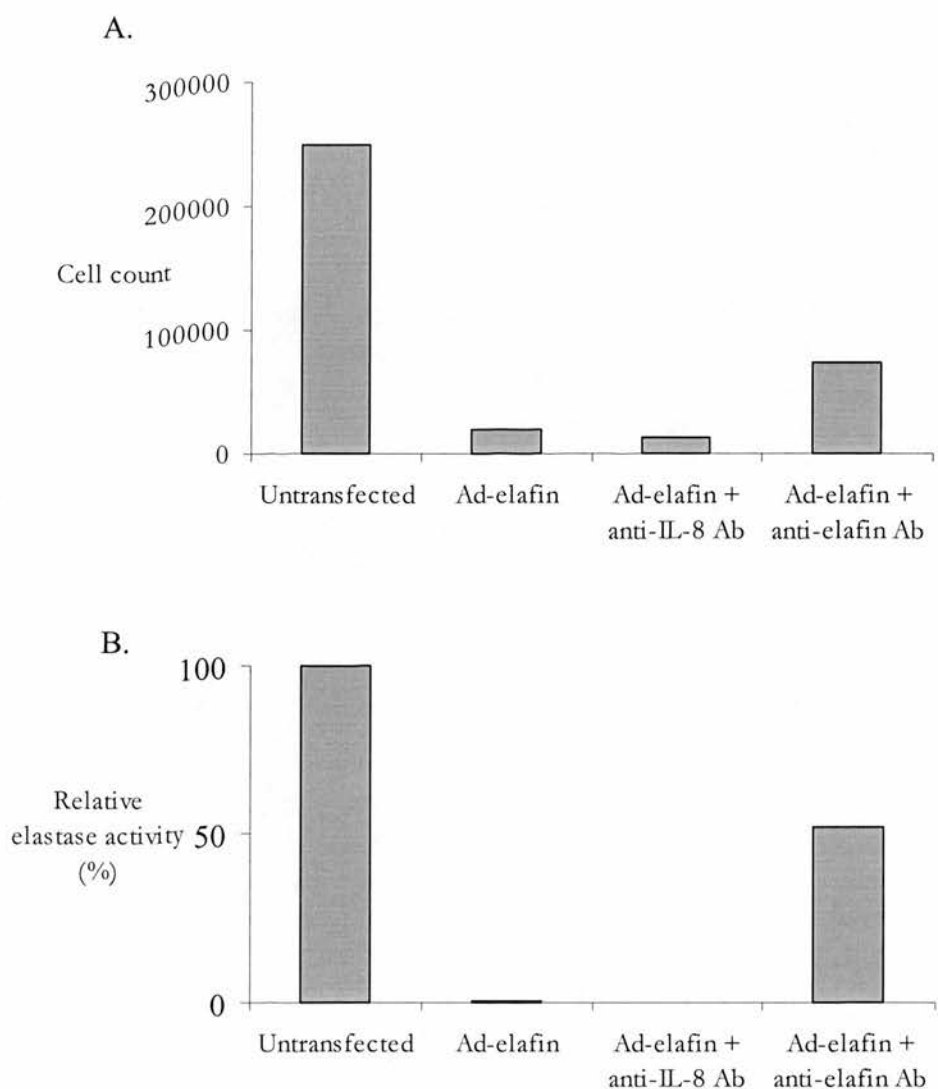


Figure 12. Ad-elafin’s protective effect against HNE-mediated damage of A549 cells is partially prevented by anti-elafin antibody.

A549 cells were treated with Ad-elafin (moi of 50) or vehicle alone, and incubated in serum-free medium for 48 hours. Ad-elafin treated cells then received an excess of anti-elafin antibody, an excess of anti-IL-8 antibody, or vehicle alone for 1 hour. All cells were then treated with HNE (final concentration 4 μ g/ml) for 16 hours. Cell counts (PANEL A) and elastase activity (PANEL B) were measured in supernatants. Elastase activity was expressed relative to the elastase activity in supernatants from untransfected cells. Data are presented as means from 2 separate experiments.

elafin was not influenced by control antibody (Figure 12, Panel B), while anti-elafin antibody resulted in partial restoration of elastase activity.

Taken together, the evidence described in this section was supportive of the hypothesis that elafin gene augmentation could protect pulmonary epithelial cells against HNE. Experiments were therefore performed to determine whether this principle could be extended to protection against the effects of whole activated neutrophils.

3.2.3. The effect of Ad-elafin transfection on neutrophil-mediated damage of A549 cells.

Preliminary experiments were performed to determine the effectiveness of neutrophil degranulation in response to activating stimuli. In the first of these PMA (1 ng/ml) was added to freshly activated neutrophils and the elastase activity in supernatants compared with that of varying concentrations of HNE at 24 hours (Figure 13). PMA stimulated 1 million neutrophils to release elastase activity similar to that associated with 4 µg/ml of HNE, suggesting effective PMA-mediated degranulation.

The preliminary findings were followed by experiments in which PMA stimulated neutrophils were applied to transfected and untransfected A549 cells. Ad-elafin transfection was associated with almost complete inhibition of released elastase activity, whilst Ad-lacZ had no effect (Figure 14). The inhibition of elastase activity was associated with morphological protection at light microscopy.

These findings stimulated experiments to determine whether similar findings were observed *in vitro* using degranulating stimuli of closer relevance to pulmonary inflammation *in vivo* (ie PAF and fMLP). Preliminary experiments were again performed to establish optimal conditions for neutrophil degranulation (Figure 15). It was determined that PAF (10^{-9} M) followed by fMLP (10^{-7} M) effected release of elastase activity at 24 hours which approximated to that effected by purified HNE at a concentration of 4 µg/ml. These concentrations of PAF and fMLP were therefore used in experiments to determine whether Ad-elafin could confer A549 cells with protection against activated neutrophils.

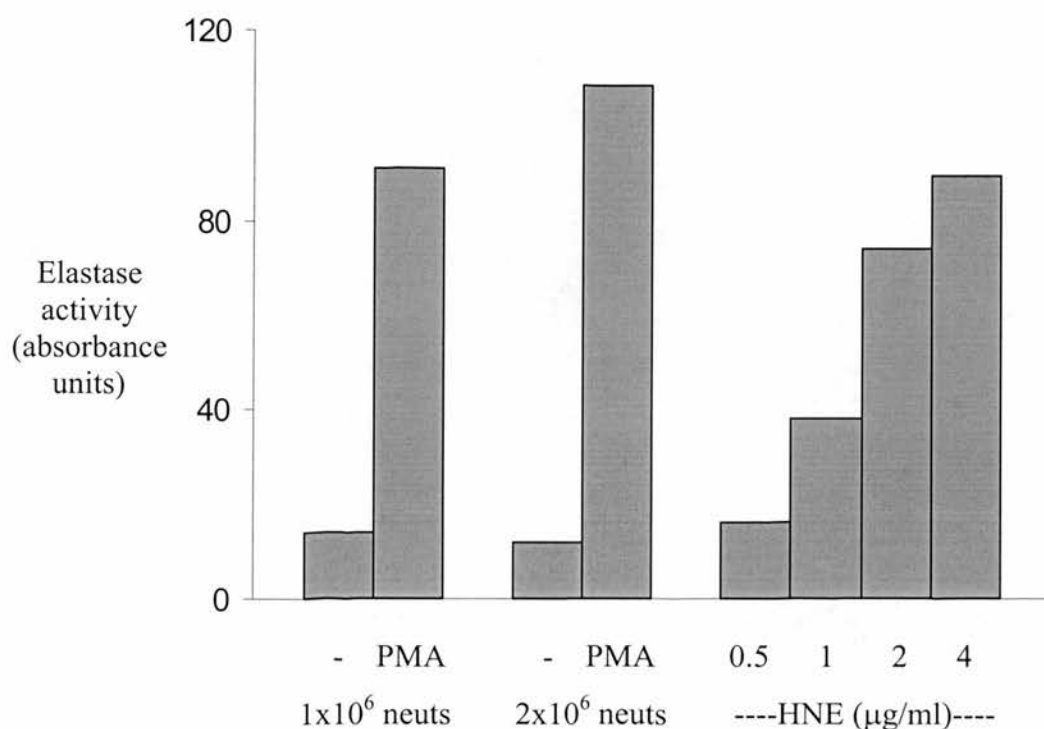


Figure 13. The effect of PMA on neutrophil degranulation.

PMA (final concentration 1 ng/ml) or vehicle alone (-) were incubated with either 1×10^6 (first two columns) or 2×10^6 (third and fourth columns) freshly prepared neutrophils (neuts) for 24 hours at 37°C. For comparison, HNE at a final concentration of 0.5-4 µg/ml (remaining four columns) was incubated in cell-free medium for the same time.

Elastase activity was determined by adding elastase-specific substrate and measuring absorbance at 405nm. PMA stimulated dose-dependent release of elastase activity from neutrophils.

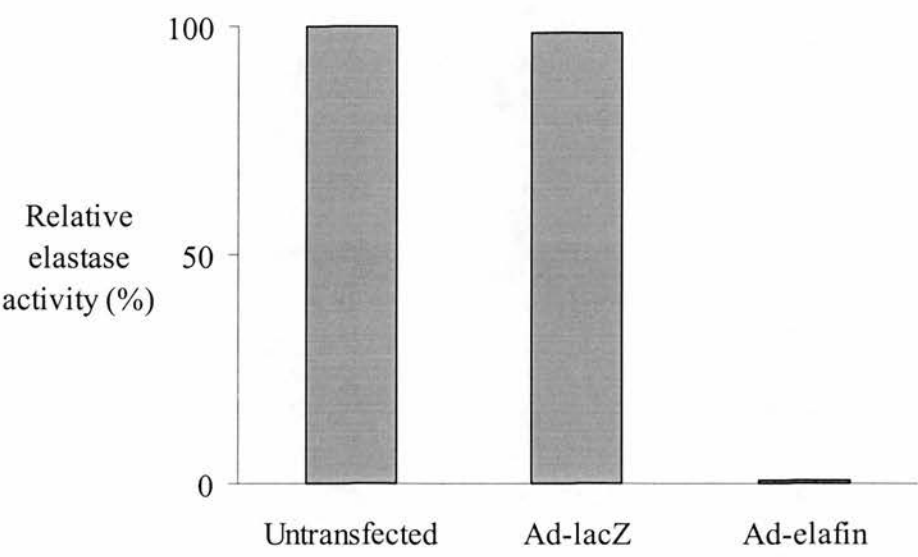


Figure 14. Transfection of A549 cells with Ad-elafin results in inhibition of HNE released by PMA stimulated neutrophils.

A549 cells were treated with Ad-elafin or Ad-lacZ (moi 50 for each) or vehicle alone, and incubated in serum-free medium for 48-72 hours prior to the addition of 1 million freshly isolated human neutrophils and PMA (final concentration 1 ng/ml). Elastase activity was measured in supernatants after 16 hours. Elastase activity was expressed relative to the elastase activity in supernatants from untransfected A549 cells.

Data represents means from 2 separate experiments.

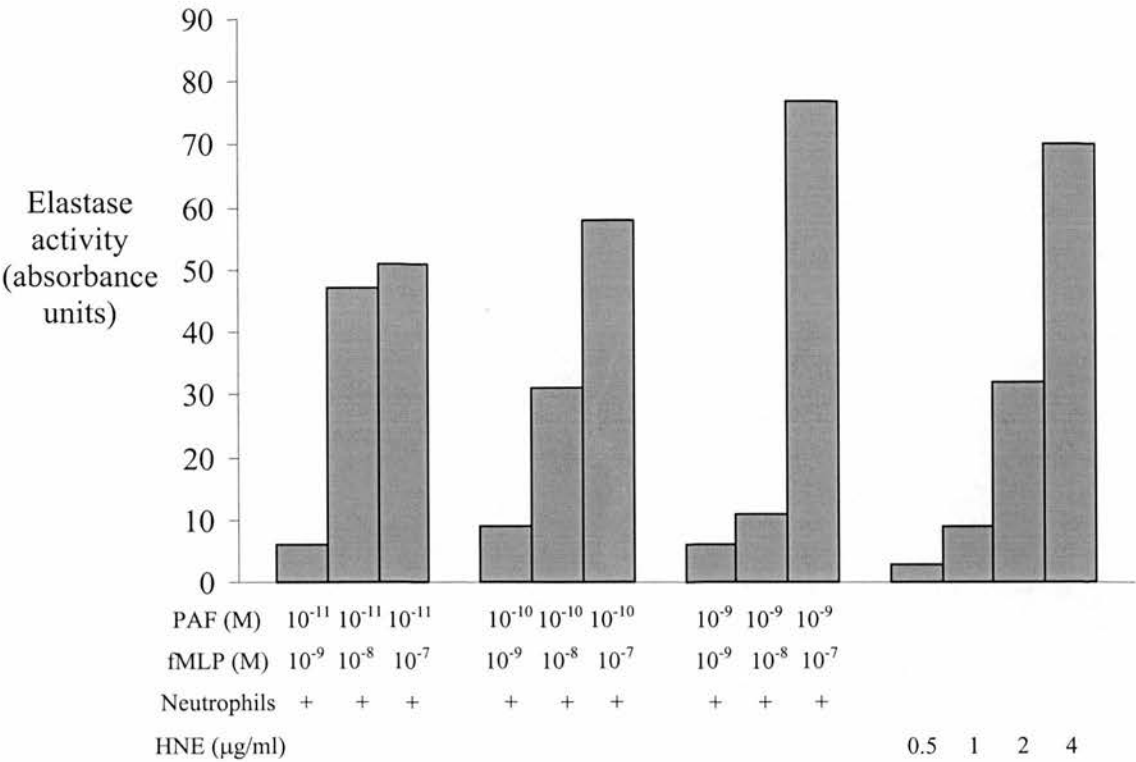


Figure 15. Neutrophil degranulation is effectively stimulated by PAF and fMLP.

Freshly isolated human neutrophils (1 million per well) were incubated at 37°C in the presence of PAF (final concentration 10^{-11} M to 10^{-9} M). After 2 hours fMLP was added (final concentration 10^{-9} M to 10^{-7} M) for a further 22 hours at 37°C. Supernatants were collected and elastase activity determined by the addition of elastase-specific substrate and measurement of absorbance at 405nm. For comparison, HNE was added to cell-free wells at 37°C, with measurement of elastase activity at 24 hours.

Results are derived from a single representative experiment.

Ad-elafin transfection was associated with considerable morphological protection of the epithelial monolayer (Figure 16). Interestingly neutrophil-mediated damage of Ad-lacZ treated (and untransfected) cells differed slightly from HNE-mediated damage in that spindling of cells was less commonly observed (compare with Figure 7). Denudation of epithelium was confined to areas populated by clusters of neutrophils (Figure 16, Panel A). Degranulation did occur in Ad-elafin treated cells, as MPO levels in medium obtained from untransfected, Ad-lacZ treated and Ad-elafin treated cells were similar (data not shown).

Neutrophil-mediated damage to A549 cells was quantified using radiolabelling methods rather than by counting cells, largely because counting was hindered by the large number of neutrophils in supernatants. ¹¹¹Indium release from Ad-elafin treated A549 cells was significantly lower than from Ad-lacZ treated cells (Figure 17). The difference in release of radiolabel between Ad-elafin treated cells and untransfected cells approached statistical significance ($p = 0.09$). Neutrophil degranulation is subject to considerable variation between donors, and this was certainly the case in the experiments described, as evidenced by the wide interquartile ranges (Figure 17). The difference in the level of ¹¹¹Indium release from untransfected cells and Ad-lacZ treated cells did not approach statistical significance ($p = 0.89$). However the higher rate of release from Ad-lacZ treated cells suggests an effect of adenovirus on radiolabel efflux. The mechanism for this adenoviral effect is unlikely to be detachment of cells as, if anything, Ad-lacZ treatment of A549 cells was associated with protection against detachment (Figure 8).

The protection provided by Ad-elafin transfection was associated with a significant inhibition of elastase activity in cell supernatants (Figure 18) suggesting that elafin augmentation was responsible for the protective effect. Elastase inhibition was almost complete using Ad-elafin at an moi of 50.

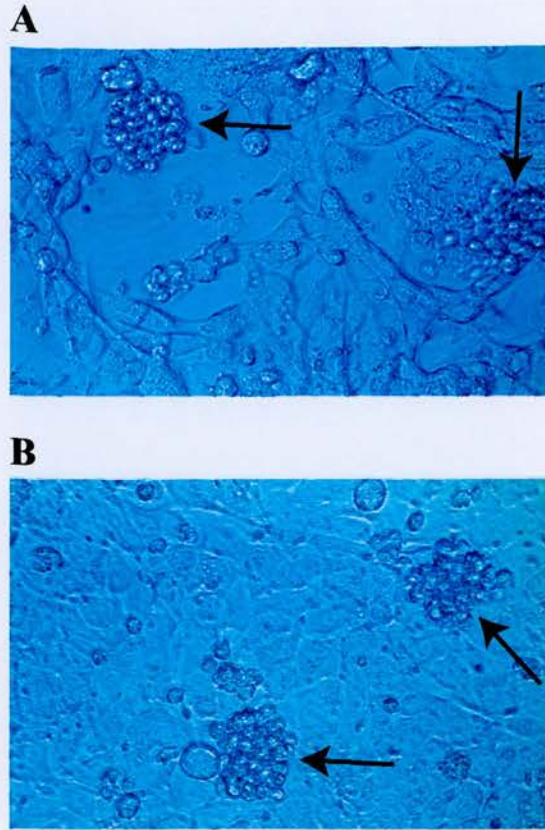


Figure 16. Transfection of A549 cells with Ad-elafin confers protection against damage mediated by neutrophil degranulation.

A549 cells were treated with Ad-elafin or Ad-lacZ (moi 50 in each case) or vehicle alone and incubated in serum-free medium for 24 hours prior to the addition of BSA and PAF (10^{-9} M). 2×10^6 freshly isolated human neutrophils were introduced, fMLP (10^{-7} M) was added 1 hour later and incubation proceeded for a further 15 hours.

PANEL A- appearance of Ad-lacZ treated cells.

PANEL B- appearance of Ad-elafin treated cells (original magnification $\times 150$ in each case).

The morphological appearance of untransfected cells closely resembled that of Ad-lacZ treated cells. The blue colour was conferred by a photographic filter used to enhance contrast. Clusters of neutrophils are indicated by arrows.

Untransfected cells to which neutrophils were not added are exemplified in Figure 7, Panel A.

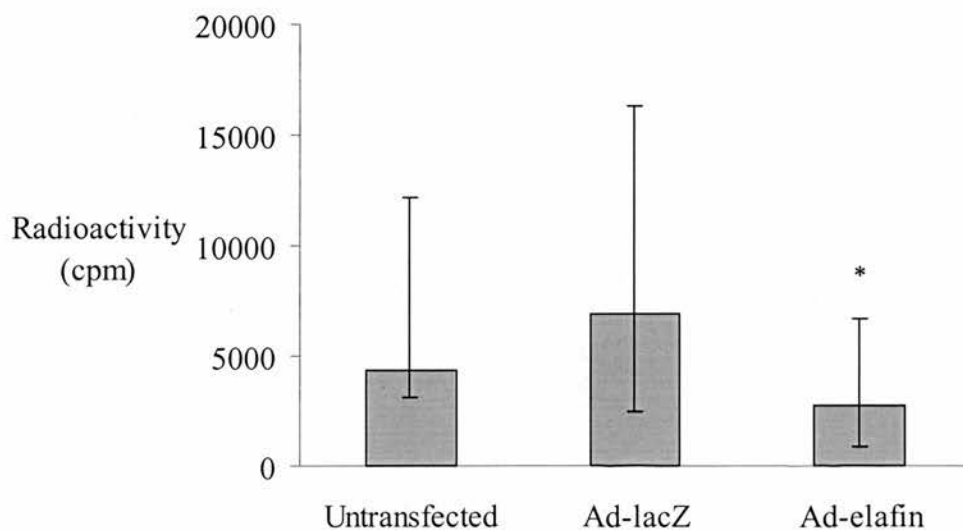


Figure 17. Transfection with Ad-elafin protects A549 cells against neutrophil-mediated damage.

A549 cells were treated with Ad-elafin or Ad-lacZ (moi of 50 in each case) or vehicle alone, then incubated with $^{111}\text{Indium}$. The cells were incubated in serum-free medium for 24 hours prior to the addition of BSA and PAF (10^{-9}M). 2×10^6 freshly isolated human neutrophils were introduced, fMLP (10^{-7}M) was added 1 hour later, and incubation proceeded for a further 15 hours. Supernatants were retrieved and radioactivity measured.

Data represent medians and interquartile ranges (n=8).

* = significant difference, $p < 0.05$, when compared with Ad-lacZ treatment.

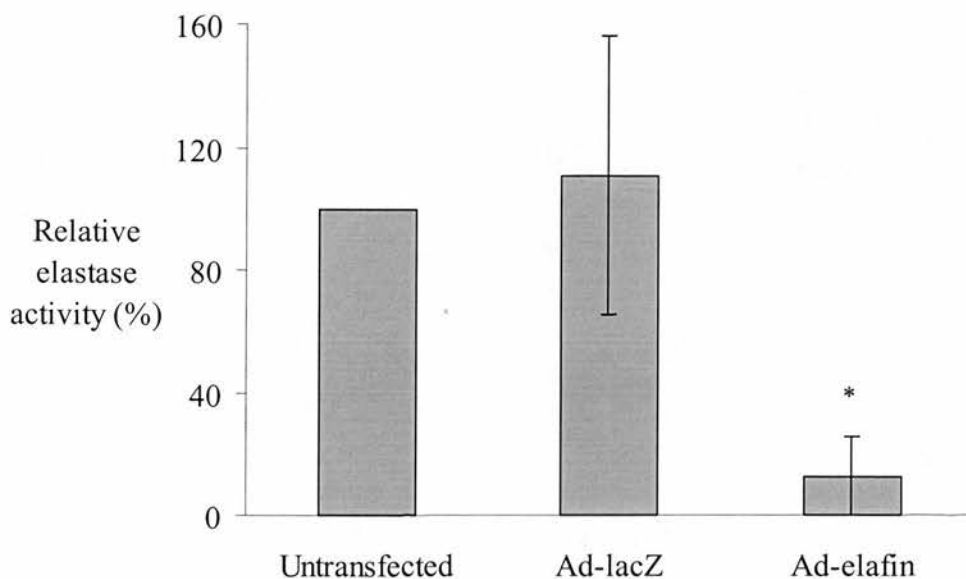


Figure 18. Transfection of A549 cells with Ad-elafin significantly inhibits the activity of elastase released by activated human neutrophils.

A549 cells were treated with Ad-elafin or Ad-lacZ (moi of 50 in each case) or vehicle alone, and incubated in serum-free medium for 24 hours prior to the addition of BSA and PAF (10^{-9} M). 2×10^6 freshly isolated human neutrophils were introduced, fMLP (10^{-7} M) was added 1 hour later, and incubation proceeded for a further 15 hours.

Supernatants were retrieved and elastase activity measured.

Elastase activity was expressed relative to the residual elastase activity in medium from untransfected cells. Data represent means and standard deviations (n=4).

* = Significant difference, $p < 0.05$, when compared with both other conditions.

3.3. DISCUSSION

The data presented in this chapter lead to three principal conclusions, namely that adenoviral augmentation of functional elafin is readily achieved in A549 cells, that genetic augmentation of elafin can protect these cells against HNE-mediated damage, and that this principle can be extended to protection against damage associated with whole, activated human neutrophils.

A549 cells were explanted from the resected right lower lobe of a 58 year old male with alveolar cell carcinoma (Giard et al, 1973), and a fundamental question therefore is whether data derived from A549 cells are relevant to the situation in the human lung? Soon after their isolation it was established that A549 cells possessed features characteristic of type II pneumocytes, including the presence of lamellar bodies and production of surfactant (Lieber et al, 1976). Importantly, serial passage did not alter the observed phenotypic characteristics (Lieber et al, 1976). Of particular relevance to the data presented here, primary human type II pneumocytes have been shown to secrete elafin (and SLPI), the secretion being up-regulated by IL-1 and TNF (Witherden et al, 1999). The latter characteristics are shared with A549 cells (Sallenave et al, 1994). Again specifically in the context of the present study, it is worth noting that the efficient adenoviral transfection of A549 cells (Figure 1) was similarly described in primary cultures of human foetal type II pneumocytes (Alcorn et al, 1997). On the basis of this evidence it seems likely that similar receptors for adenovirus entry are present on type II pneumocytes and A549 cells. Against this background, and given both the extensive international laboratory experience with A549 cells and their ready accessibility, it was felt that A549 cells were plausible surrogates for type II pneumocytes *in vitro*.

The data presented in Figure 4 confirm the finding that native (untransfected) A549 cells constitutively generate EIA. It was already known that A549 cells could produce SLPI, elafin and α_1 -PI (Sallenave et al., 1993; Venembre et al., 1994; Sallenave et al., 1997b). The observation that elafin makes a small contribution to measured EIA is broadly in keeping with the finding that A549 cells produce 25 times more SLPI than elafin (Sallenave et al., 1993). Interpretation of the data presented in this chapter with regard to the contribution of elafin to EIA should be tempered by the fact that A549 cells were cultured in serum-free medium (because of the high concentration of elastase inhibitors in serum). It could be argued that the lack of nutrients may have altered generation of EIA. While this cannot be entirely

excluded, secretion of elafin antigen from untransfected (and Ad-lacZ transfected) A549 cells was similar in the presence and absence of serum (for comparison, data relating to serum-containing medium is shown in Chapter 4, Figures 12 and 13, left hand columns).

The finding that Ad-lacZ did not increase elafin or EIA secretion from A549 cells (Figures 3 and 4) has fundamental implications for the data in this chapter. There is little doubt that the phenotype of A549 cells may be altered by viral infection. Respiratory syncytial virus (RSV) stimulates secretion of IL-6, IL-8 and soluble TNF receptor type I from A549 cells (Arnold et al., 1994) and adenoviral infection increases expression of ICAM-1 from A549 cells (Stark et al., 1996). It is interesting therefore that Ad-lacZ did not up-regulate secretion of EIA or elafin (indeed, if anything there was a small decrease in secretion of each). The encouraging message is that the marked augmentation of elafin secretion after transfection with Ad-elafin is attributable to the transgene and not to viral infection *per se* (Figures 2 and 3). It was similarly encouraging that the elafin produced was associated with significant anti-elastase activity (Figure 4).

The considerable genetic augmentation of elafin described was sufficient to ameliorate HNE-induced damage of A549 cells (Figures 7,8 and 9). Synthetic inhibitors of elastase have previously been shown to protect A549 cells against HNE (Ayars et al., 1984), and genetic augmentation of α_1 -PI using liposomal gene transfer extended the principle in a CF bronchial epithelial cell line (Canonico et al., 1996). Furthermore, differentiated human keratinocytes which secrete elafin in response to biochemical stimuli are protected against detachment induced by very high doses (10 μ g/ml) of HNE (Pfundt et al., 1996). However the data in this chapter are the first to describe protection of pulmonary epithelial cells conferred using adenoviral augmentation of an endogenous pulmonary elastase inhibitor. Interestingly, the concentration of HNE which could be inhibited by Ad-elafin transfection is within the range of concentrations found in BALF from patients with pneumonia (Boutten et al., 1996). Certain aspects of the data describing inhibition of HNE by Ad-elafin merit closer attention.

In keeping with the findings here (Figure 8), detachment has been the predominant injury induced in A549 cells by HNE in other studies (Ayars et al., 1984; van Wetering et al., 1997). However a propensity to HNE-mediated cell lysis was also described in adherent untransfected and Ad-lacZ transfected cells in this

study, the effect being markedly inhibited by Ad-elafin transfection. Interestingly Ayars et al. (1984) noted that detachment was the predominant process at 1 $\mu\text{g/ml}$ of HNE, but that lysis predominated at concentrations of 10 $\mu\text{g/ml}$ and above. In summary, in this study Ad-elafin transfection appeared to protect against both detachment and lysis, in that the epithelial monolayer remained relatively intact and, more importantly, the remaining adherent cells remained viable.

The results described here differ in some respects from those in the careful characterisation of HNE-mediated damage performed by Ayars et al. (1984) in that higher doses of HNE required to be applied for longer to effect damage in this study (Figure 6). In this context it is surprising that detachment was not invariably seen when concentrations of HNE of 1-2 $\mu\text{g/ml}$ were applied to untransfected cells. Given that the HNE used was approximately 50% active these conditions predict for 16-33 nM HNE and approximately 0.8 nM of EIA (Figure 4). In the same way it is interesting that Ad-elafin transfection (generating approximately 9.1 nM of EIA) effectively inhibited 4 $\mu\text{g/ml}$ (approximating to 66 nM) of HNE. This raises the intriguing possibility that cellular (and/or matrix) targets neutralise a proportion of the HNE applied.

In this context it is interesting that while Ad-elafin transfection effected rapid and almost complete inhibition of HNE in cell supernatants (Figure 6), damage to the cells (albeit limited) still occurred by 16 hours (Figures 7 and 8). It remains plausible that a proportion of the HNE comes into contact with intercellular attachments and in so doing evades detection by elafin and effects a gradual proteolytic detachment. In favour of such a hypothesis is the observed potential for elastase to bind connective tissue in humans and hamsters (Watanabe et al., 1990; Rudolphus et al., 1992), and the relative resistance of bound HNE to inhibition (Morrison et al., 1990).

A further interesting observation was the relative reduction in elafin detected by ELISA after addition of HNE to Ad-elafin treated cells (Figure 10). One potential explanation is that the anti-elafin antibody used is less capable of detecting pre-formed elafin-HNE complex; the antibody was raised against a COOH-terminal fragment of elafin which may potentially be masked upon binding with HNE. An alternative explanation may lie in the observed reduction of elafin secretion from A549 cells stimulated with HNE (Reid et al., 1999). A further potential explanation may be proteolytic degradation of elafin by HNE, though this may be less likely

given that elafin is known to retain almost full activity upon dissociation with HNE *in vitro* (Ying and Simon, 1993).

The fact that protection conferred by Ad-elafin transfection was associated with an increase in elafin antigen and an increase in anti-elastase activity suggested that elafin was the moiety directly responsible for protection against HNE. Interestingly, neutralisation of elafin using anti-elafin antibody resulted in a partial (but incomplete) restoration of HNE-mediated cell detachment and HNE activity (Figure 12). It therefore appears that elafin is, at least in part, directly responsible for the protection observed. Two possibilities exist to explain the residual inhibition of HNE-mediated damage after incubation with an excess of anti-elafin antibody. The first is that optimal conditions did not exist for binding of the high concentrations of antibody used to elafin in the complex conditioned medium described here (Figure 12). The second is that an alternative moiety (which must by definition have anti-elastase activity) is induced. In my opinion the former explanation seems more likely, for a number of reasons. Firstly, a putative anti-elastase would require to be induced specifically by elafin (as no such inhibition was observed in Ad-lacZ treated cells). In the only study to date in which SLPI levels were quantified in association with markedly augmented levels of elafin from A549 cells, only a modest rise in SLPI levels was detected (Sallenave et al., 1994). In addition, the rise in EIA generated by Ad-elafin transfection in this study can be explained by an increase in elafin alone. Finally, while the anti-elafin antibody was proven to be effective in serum-free DMEM in a cell-free system *in vitro*, it may have been less effective in conditioned medium containing HNE. Establishing optimal conditions for antibody binding can be notoriously difficult in complex media, and HNE itself is capable of degrading immunoglobulins *in vitro* (Baici et al., 1980).

Irrespective of the issues raised above, this chapter provides unequivocal evidence for a protective effect of Ad-elafin against HNE-mediated damage of A549 cells. This provided the impetus to examine whether this principle could be extended to protection against neutrophil-mediated damage. Significant degranulation of neutrophils was achieved, using mechanistically distinct agents in the form of PMA (Figure 13) and a combination of the priming agent PAF and the secretagogue fMLP (Figure 15). Protection of A549 cells by Ad-elafin transfection was demonstrated at light microscopy whether PMA or PAF/fMLP was used as the stimulus to degranulation (appearances after PMA stimulation closely resembled those in Figure 16 in which degranulation was mediated by PAF/fMLP stimulation), the protection

being associated with almost complete inhibition of HNE (Figures 14 and 18). Protection against the effects of neutrophil degranulation may be considered surprising at first glance, given that elafin is known to inhibit specifically only two neutrophil proteases (HNE and proteinase 3), leaving an array of potentially cytotoxic degranulation products to damage cells (as discussed in section 1.3.2.).

However previous studies have demonstrated that elastase inhibitors are significantly more effective than anti-oxidants in ameliorating neutrophil mediated damage to microvascular endothelial cells from adipose tissue (Smedley et al., 1986), human pulmonary artery endothelial cells (Furuno et al., 1997) and A549 cells (Ayars et al., 1984). In addition keratinocytes which do not secrete elafin could be markedly protected against neutrophil-mediated detachment by (extremely high doses of) recombinant elafin (50 µg/ml), whilst anti-oxidants had little effect (Pfundt, et al., 1996).

The minimal effect of anti-oxidants in the studies cited above is intriguing given the propensity for neutrophils to release activated oxygen species upon degranulation (reviewed by Weiss, 1989). The important implication for this study is that, despite elafin containing a methionine in its reactive site (Tsunemi et al., 1996) and therefore being susceptible to inactivation by changes in redox potential (Tsunemi et al., 1992), Ad-elafin can still generate anti-elastase activity in complex, cell-containing media. This may have important consequences as SLPI and α_1 -PI can both be significantly inactivated by PMA-stimulated human neutrophils (Vogelmeier et al., 1997). Exactly how elafin's reactive site evaded oxidation is uncertain, though it is clear that A549 cells are capable of constitutively producing anti-oxidants such as glutathione (Li et al., 1994; Järvinen et al., 2000).

As in the case of HNE-mediated damage, it is instructive to consider the effects of activated neutrophils in damaging susceptible cells and their interstitium, be those cells transfected with adenovirus or not. Approximately 98% of damage to A549 cells by PMA-activated neutrophils has been shown to be due to detachment, with the other 2% attributable to lysis (Ayars et al., 1984). As was the case for HNE-mediated damage, Ayars et al. (1984) described much more rapid neutrophil-mediated damage of untransfected A549 cells than was observed here. In contrast, Wang et al. (1998) described a time course more in keeping with that observed here. The latter group extended their studies to examine the effect of RSV infection on susceptibility to neutrophil damage. They found that RSV *per se* was associated with

A549 cell damage, and that RSV potentiated the damaging effects of neutrophils (Wang et al., 1998). The relevance of this observation to the present study is that the adenovirus-mediated up-regulation of ICAM-1 referred to previously (Stark et al., 1996) may enhance neutrophil adhesion to A549 cells. This mechanism may in part explain the augmented (though statistically non-significant) neutrophil-mediated damage to Ad-lacZ treated cells as compared with untransfected cells in Figure 17.

Certainly neutrophil adhesion appears to be important in effecting damage of neutrophil substrate. In this context it is worth observing that neutrophil-mediated damage of labelled fibronectin has been shown to occur in a characteristically pericellular distribution (Campbell and Campbell, 1988). A strikingly pericellular distribution of damage around clusters of neutrophils was observed in this study also (Figure 16). The concept of adhesion-dependent neutrophil-mediated damage subsequently led to the discovery that elastase could be bound and concentrated at the neutrophil membrane (Bangalore and Travis, 1994; Owen et al., 1995). This consolidated interest in data showing that α_1 -PI was excluded from the critical space between membrane and substrate (Campbell and Campbell, 1988). Certainly it was demonstrated that membrane bound HNE is less susceptible to inhibition by endogenous anti-elastases than is the free form (Bangalore and Travis, 1994). In general it has emerged that elastase inhibitors of low molecular weight and net positive charge may be best able to access membrane bound HNE (Owen et al., 1995). Against this background it seems possible that elafin, as a low molecular weight cationic inhibitor of elastase, may be particularly well suited to inhibiting membrane bound HNE. Whether this mechanism contributes to the observed protective effect of Ad-elafin remains unknown.

One study has suggested that neutrophil granule fractions containing defensins (rather than HNE) account for neutrophil-mediated damage to A549 cells (Okrent et al., 1990). While neither the concentration nor the activity of HNE in the relevant granule fraction was determined in the study by Okrent et al. (1990), it is hard to ignore the effect of the defensin fraction. Subsequent studies have indicated that HNE and defensins have quite separate destructive capabilities towards A549 cells, HNE generally inducing detachment and defensins inducing lysis (van Wetering et al., 1997). Intriguingly, neutrophil defensins inhibit HNE-mediated detachment of A549 cells, and HNE inhibits defensin-mediated lysis (van Wetering et al., 1997). Taken together, these strands of evidence could be interpreted as suggesting that the protective effect of Ad-elafin in this study is not related to

inhibition of extracellular HNE, but instead is due either to an effect on defensin release and/or to a specific effect on degranulation. A specific effect of elafin on degranulation is unlikely, as MPO was easily detected in supernatant from Ad-elafin treated cells in a representative experiment. Whether elafin augmentation influences defensin biology is unproven. Conversely it seems that defensins have no significant effect on elafin production in primary bronchial epithelial cells (van Wetering et al., 2000). On balance it must be concluded that more evidence exists to support an anti-elastase mechanism for the protection afforded against activated neutrophils by Ad-elafin. This conclusion is reached on the basis of reduced elastase activity in supernatants from Ad-elafin treated cells (Figure 18), and the striking resemblance of results derived using HNE in place of neutrophils (compare Figures 7,9 and 11 with Figures 16,17 and 18).

Taken together the results of this chapter indicate that genetic augmentation of functional elafin in pulmonary epithelial cells is feasible, and that protection can be provided against HNE- and neutrophil-mediated damage. The potential extrapolation of the protective data to *in vivo* situations must be partially tempered by recognition of the fact these experiments were necessarily performed using A549 cells cultured in serum-free medium. None the less the results are strongly supportive of the concept that HNE plays a critical role in neutrophil-mediated injury of tissues (Smedley et al., 1986; Pfundt et al., 1996; Furuno et al., 1997). They furthermore provide the necessary proof of principle to proceed towards studies of the effects of Ad-elafin on conditions characterised by pulmonary neutrophil influx *in vivo*.

CHAPTER 4

THE EFFECT OF ELAFIN GENE AUGMENTATION ON AIRWAY NEUTROPHILIA INDUCED BY LPS IN MICE

4.1. AIMS

The demonstration of Ad-elafin's protective effect against neutrophil-mediated damage *in vitro* stimulated the hypothesis that Ad-elafin may confer tissue protection in inflammatory lung disorders characterised by neutrophil influx and generation of free elastase. In identifying conditions for which Ad-elafin might be specifically targeted it was first necessary to determine the effect of elafin augmentation on pulmonary neutrophil extravasation, given that elastase may play a role in this process.

Against this background a fundamental aim of this chapter was to determine whether Ad-elafin transfection could generate human elafin in murine airways. Assuming this target to be achievable, the principal aim was to determine the effect of elafin augmentation on acute pulmonary neutrophilia induced by LPS.

As discussed in section 1.7.4., adenovirus itself induces pulmonary inflammation, and in this context a further aim was to characterise and minimise vector-induced inflammation in the murine lung.

The remaining aim of the work in this chapter was to characterise the effect of LPS on Ad-elafin secretion from transfected cells *in vitro*.

4.2. RESULTS

4.2.1. Normal composition of BALF in C57/Bl6 mice.

Two key points of reference must be made in prefacing the data in this chapter, namely the cell count and protein concentration in BALF retrieved from normal mice. Four healthy C57/Bl6 mice were killed and BALF retrieved - the median cell count was 184,000, 98% of which were alveolar macrophages, and 2% neutrophils. The median protein concentration in BALF was 0.45 g/l. No human

elafin was detected in BALF from any of the mice in keeping with experiments previously performed by Dr Jean-Michel Sallenave, in which the anti-human elafin antibody used in this work showed no cross-reactivity with murine serum or BALF. The BALF from mice treated IT with PBS alone shows a similar cellular composition and protein concentration to BALF retrieved from normal, untreated mice. Data described throughout the remainder of this work will illustrate that the macrophage is the predominant cell type in normal BALF regardless of the fact that total cell numbers are subject to considerable variability. For the purposes of this study, based on my experience in the lab, the "normal" cell count in mice exposed to vehicle alone is considered to be approximately 200,000 with a normal range of 80,000-400,000, over 95% of which should be alveolar macrophages. A normal BALF protein concentration is considered to be less than 0.55 g/l.

The need to establish these reference values related to the importance of determining the degree of adenovirus-induced neutrophilia in BALF in subsequent experiments. Given that one of the principal aims of this chapter was to determine the effect of adenoviral augmentation of elafin on LPS-induced neutrophilia, it became desirable to effect elafin expression with the minimal degree of vector-induced inflammation possible.

4.2.2. Intranasal and intraperitoneal administration of Ad-elafin.

Initial experiments employed the IN route to determine the effects of Ad-elafin administration in murine airways (Table 1). Human elafin was undoubtedly generated in murine airways, none of which was found circulating in serum from any of the mice studied. Neither elafin generation nor increasing dose of adenovirus were associated with a significant elevation in the total cell count in BALF (Table 1). However a considerable degree of variability was inherent in the system. In particular, no elafin could be detected in BALF from 4 of the mice studied. This led to concerns relating to the reproducibility of vector distribution using the IN route. Three mice therefore received an IN dose of trypan blue, followed 4 hours later by sacrifice and retrieval of lungs. Macroscopic examination revealed that dye was confined to the apical regions of the lungs, with no staining of the bases; in two mice staining was visible in the stomach, indicating that a proportion of the dose was swallowed. Furthermore the apical staining in the lungs was variable in its intensity in different mice, lungs from one of the mice having virtually no macroscopic staining.

Dose of Ad-elafin	DAY 5			DAY 9		
	3x10 ⁷ pfu	3x10 ⁸ pfu	1x10 ⁹ pfu	3x10 ⁷ pfu	3x10 ⁸ pfu	1x10 ⁹ pfu
Total cell count in BALF	98200	184500	105000	154000	220000	261000
BALF elafin (ng/ml)	4.9	0	26.2	5.0	27.5	0
Serum elafin (ng/ml)	0	0	0	0	0	0

Table 1. Intranasal administration of Ad-elafin results in variable expression of human elafin in BALF.

C57/Bl6 mice received an intranasal instillation of Ad-elafin (3x10⁷ pfu, 3x10⁸ pfu or 1x10⁹ pfu) in a total volume of 30µl. Mice were killed either 5 days later or 9 days later. Serum was retrieved and the human elafin concentration measured. BALF was also retrieved and the total white cell count and human elafin concentration estimated. In 3 separate animals receiving 30µl of PBS intranasally and killed on days 5-9 the average cell count in BALF was 233,300, and no human elafin was detected in either BALF or serum.

Results represent medians (n=3 in each group).

On the basis of these findings it was concluded that human elafin could be generated in murine airways for at least 9 days but that the variability of elafin expression, along with the strikingly regional distribution of vector, precluded IN administration for experiments studying the effect of elafin augmentation on pulmonary neutrophilia.

Studies were also performed using the IP route, principally with the aim of generating circulating elafin to determine whether transfer to the airspaces could be achieved (Table 2). Elafin was only detectable in serum from 3 of 18 mice (one receiving 3×10^8 pfu, the other 2 receiving 1×10^9 pfu). The 3 mice in question were killed on day 5. No elafin was detected in serum from any of the 6 mice studied on day 9. No elafin was detected in BALF from any of the animals sacrificed on day 5 or day 9. These data made it clear that an alternative means of administration would be required to effect consistently reliable expression of human elafin in murine airways.

4.2.3. Intratracheal administration of Ad-elafin.

Three mice received an IT instillation of trypan blue. In marked contrast to the distribution of dye after IN administration, dye was distributed to each lobe of both lungs, with minimal variation between animals. No dye was seen macroscopically in stomach. Varying doses of Ad-elafin were therefore instilled IT, and elafin levels were measured in BALF over the course of 8 days (Figure 1). Elafin was detected in BALF from 26 of the 27 animals studied. Interestingly, low dose Ad-elafin resulted in expression of human elafin without an increase in either total cell count or neutrophil count when compared with PBS treated mice. Although elafin expression was generally more efficient at higher doses of vector, these were (with the exception of BALF from mice treated with high dose Ad-elafin retrieved on day 8) associated with varying degrees of airway neutrophilia.

It is unclear why high dose (1×10^9 pfu) administration resulted in lower levels of elafin expression and less pronounced BALF leukocytosis than was observed after administration of an intermediate dose (3×10^8 pfu). This unexpected finding is returned to in the discussion (section 4.3.1.1.) but does not detract from the principal conclusions derived from these experiments, that low-dose Ad-elafin (3×10^7 pfu) could consistently generate human elafin in murine airways without inducing overt airway neutrophilia. Although the elafin concentrations generated

	DAY 5				DAY 9		
	3x10 ⁷ pfu	3x10 ⁸ pfu	1x10 ⁹ pfu	3x10 ⁷ pfu	3x10 ⁸ pfu	1x10 ⁹ pfu	
Dose of adenovirus							
Total cell count in BALF	125000	238000	252500	195000	310000	228000	
BALF elafin (ng/ml)	0	0	0	0	0	0	
Serum elafin (ng/ml)	0	0	4.2	0	0	0	

Table 2. Intraperitoneal administration of Ad-elafin does not result in expression of human elafin in BALF.

C57/Bl6 mice received an intraperitoneal instillation of Ad-elafin (3x10⁷ pfu, 3x10⁸ pfu or 1x10⁹ pfu) in a total volume of 200µl. Mice were killed either 5 days later or 9 days later. Serum was retrieved and the human elafin concentration measured. BALF was also retrieved and the total white cell count and human elafin concentration estimated.

Three separate animals received 200µl of PBS intraperitoneally and were killed on days 5-9. The average cell count in BALF was 205,000, and no human elafin was detected in either BALF or serum.

Results represent medians (n=3 in each group).

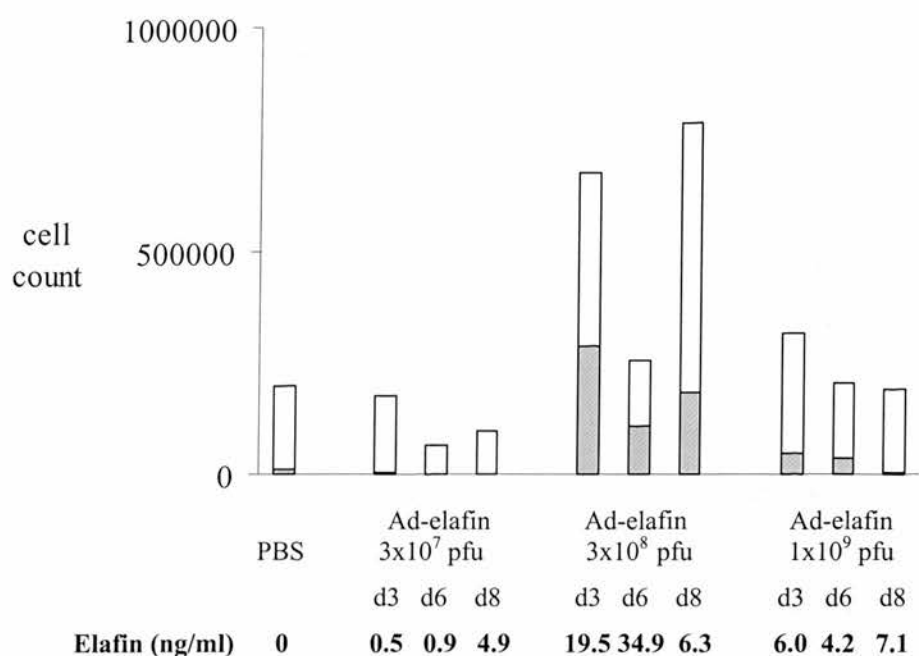


Figure 1. Intratracheal administration of Ad-elafin generates human elafin in BALF, but at higher doses of vector this is accompanied by significant vector-associated neutrophilia.

C57/Bl6 mice received an IT instillation of Ad-elafin at a dose of either 3×10^7 pfu, 3×10^8 pfu, or 1×10^9 pfu ($n=9$ in each group). Within each group, mice were killed at day 3, day 6 or day 8 ($n=3$ in each subgroup). Two mice received PBS IT; one was sacrificed at 24 hours, one at 48 hours.

BALF was retrieved from each mouse. Total cell count, neutrophil count and elafin concentration in BALF were estimated.

Columns represent median cell counts in BALF. Shading indicates the number of neutrophils, open boxes indicate the number of cells other than neutrophils. Median elafin concentrations in BALF are indicated in bold print.

using this dose were relatively low, it may be assumed that higher local concentrations are generated in epithelial lining fluid (and possibly at tissue level). The absence of vector-induced neutrophilia was considered critical given that the principal aim of this chapter was to determine the effect of Ad-elafin transfection on LPS-induced neutrophilia. For these reasons it was decided that low dose Ad-elafin should be used in experiments to determine the effect of elafin gene augmentation on LPS-induced pulmonary neutrophilia.

4.2.4. Experiments to establish a dose of LPS generating moderate airway neutrophilia.

Further preliminary experiments were required to establish a dose of LPS which would generate moderate airway neutrophilia. Given that there was no specific preliminary data to indicate whether elafin augmentation was likely to augment or ameliorate LPS-induced neutrophilia (if indeed it was to have any effect at all), then the degree of neutrophilia induced in control mice needed to be sensitive to a change in either direction. That is, moderate neutrophilia was required, as a striking LPS-induced neutrophilia would preclude detection of any augmentation by elafin, and a low-grade neutrophilia would preclude detection of any inhibition by elafin.

Experiments were therefore performed to establish the time-course and dose-dependence of LPS-mediated neutrophilia (Figures 2 and 3). IT LPS (7.5µg) caused a progressive and striking rise in BALF neutrophil count over 48 hours, with a corresponding rise in MIP-2 concentrations in BALF (Figure 2). On the basis of this experiment it was concluded that a lower dose of LPS was required, but that augmentation of neutrophil count was likely to be achievable by 24 hours even with lower doses of LPS. Therefore mice were given either 0.1µg or 0.5µg of LPS IT, and killed at 24 hours. Whilst 0.1µg resulted in a low-grade neutrophilia, 0.5µg resulted in a moderate neutrophilia, neutrophils comprising approximately 50% of the total cells in BALF (Figure 3).

4.2.5. The effect of Ad-elafin on LPS-induced airway neutrophilia.

The results described thus far indicated that Ad-elafin (3×10^7 pfu delivered IT) could consistently generate human elafin in murine airways for at least 8 days without inducing neutrophilia, and that IT LPS at a dose of 0.5µg could generate a

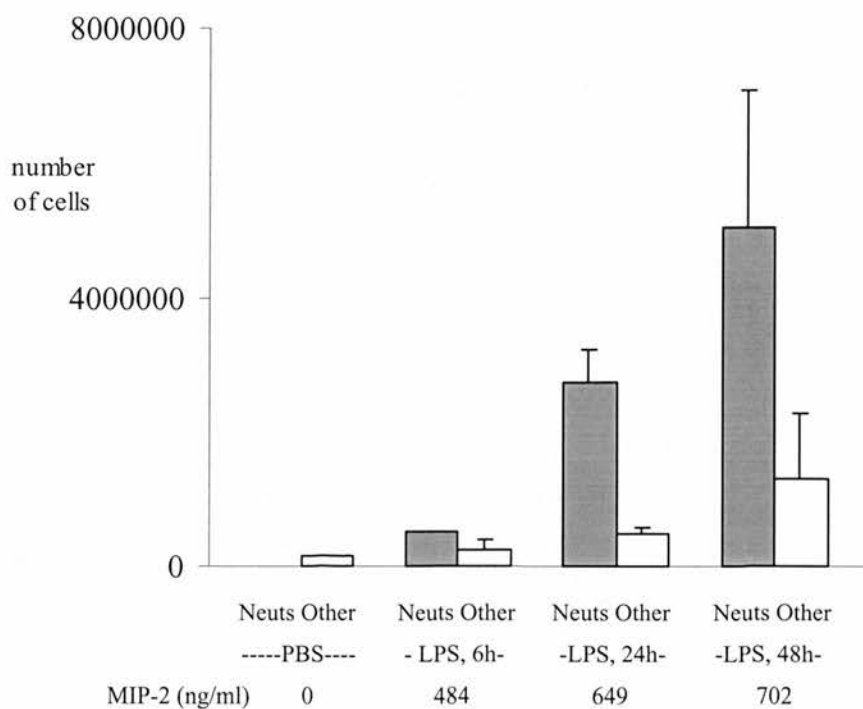


Figure 2. Intratracheal LPS administration results in a time-dependent increase in airway neutrophilia.

C57/Bl6 mice received an IT instillation of 7.5 μ g of LPS and were killed either 6, 24 or 48 hours later. One representative mouse received PBS via the IT route and was killed at 48 hours. Total cell count, neutrophil count and MIP-2 concentrations were established in BALF.

Neutrophil count ('Neuts') in BALF is indicated by shaded boxes, and the remaining number of cells in BALF ('Other') is indicated by open boxes. Results represent means and standard deviations (n=4 per group except for PBS, where n=1).

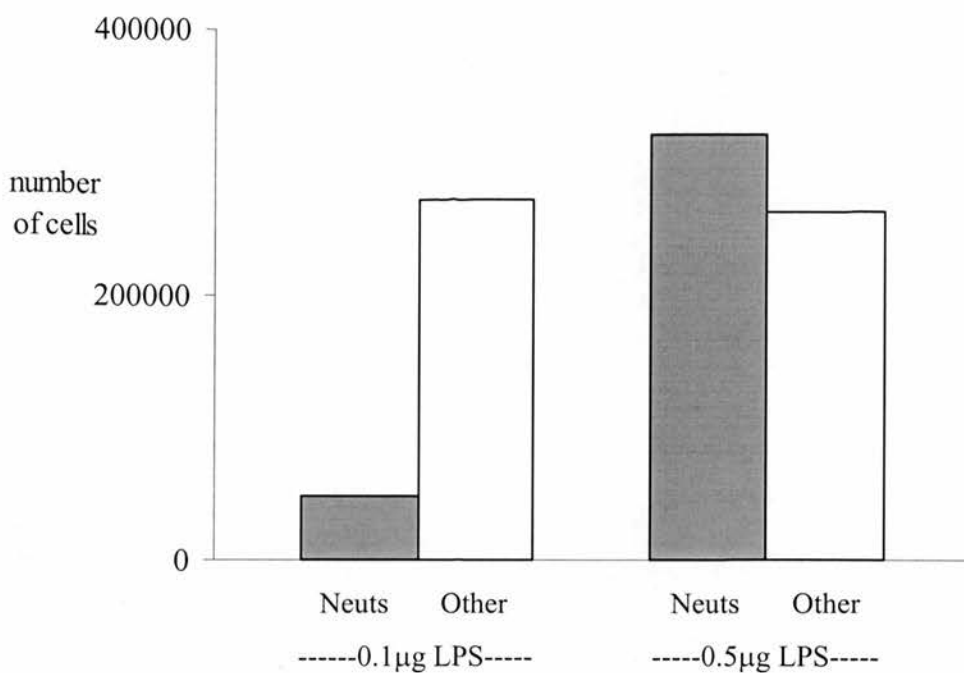


Figure 3. Intratracheal administration of LPS results in a dose-dependent increase in airway neutrophilia.

C57/Bl6 mice received an IT instillation of either 0.1 or 0.5 µg of LPS (n=2 in each group) and were killed 24 hours later. Total cell count and neutrophil count were established in BALF.

Neutrophil count ('Neuts') in BALF is indicated by shaded boxes, and the remaining number of cells in BALF ('Other') by open boxes. Results are presented as the average cell count for the 2 mice in each group.

moderate airway neutrophilia at 24 hours. These conditions satisfied the necessary criteria to study the effect of Ad-elafin on LPS-induced neutrophilia *in vivo* using the experimental protocol described in Table 3.

In assessing the effect of Ad-elafin on LPS-mediated neutrophilia it was considered necessary to determine whether adenoviral transgene expression was achieved in the distal lung, given that migration of neutrophils into airways is most likely to proceed in and around distal airspaces. Figure 4 demonstrates that alveolar and bronchial epithelium (as well as alveolar macrophages) were accessed by adenovirus under the experimental conditions described in Table 3.

Interestingly, Ad-elafin resulted in a striking and statistically significant augmentation of the airway neutrophilia associated with LPS (Figure 5). The enhancement of neutrophil count was similarly represented in cytopspins derived from BALF (Figure 6). The effect did not appear to be confined to "lavageable" neutrophils loose in the airways, as a similar pattern of appearance was observed in histological sections (Figure 6). A small (statistically non-significant) rise in total macrophage count was also observed in Ad-elafin/LPS mice.

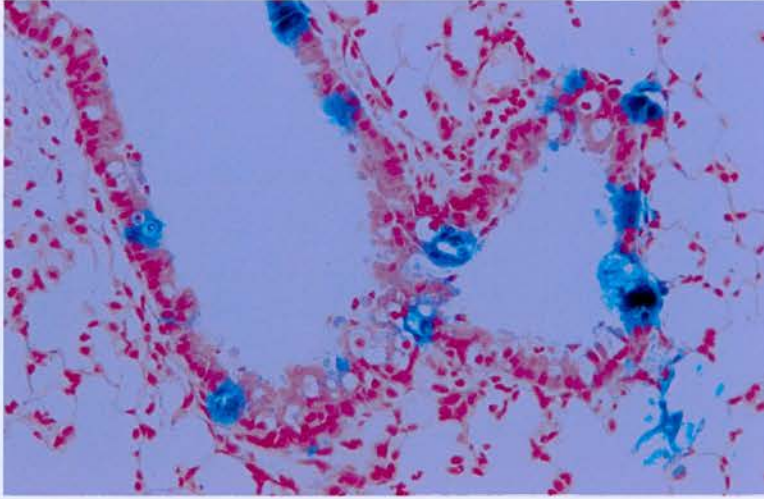
Importantly the enhancement of airway neutrophilia in Ad-elafin/LPS mice was not associated with significant disruption of the alveolar-capillary membrane, as BALF protein concentrations were lower in Ad-elafin/LPS mice than in either of the other groups receiving LPS despite more than a doubling in neutrophil migration (Figure 5). Interestingly, the neutrophilia induced by LPS in the absence of Ad-elafin was associated with minor disruption of the alveolar-capillary membrane, there being only a small increment in BALF protein concentration when comparing PBS/PBS mice with PBS/LPS mice or Ad-lacZ/PBS mice with Ad-lacZ/LPS mice (indeed a similar pattern was also observed when comparing Ad-elafin/PBS mice with Ad-elafin/LPS mice) (Figure 5). For each comparison the small increment in BALF protein concentration was not statistically significant, and for all groups studied in Figure 5 protein concentrations generally remained in (or just above) the normal range. Thus it appears that in the model studied, LPS-mediated neutrophilia induces little or no lung injury at 24 hours, while the augmentation of LPS-induced neutrophilia associated with Ad-elafin treatment is not attended by alveolar-capillary membrane disruption.

n	DAY 0	DAY 5	DAY 6
4	PBS	PBS	Mice killed for retrieval of... SERUM MIP-2 and elafin concentration. BALF For total cell count, differential cell count, cytospin. Concentrations of elafin, MIP-1 α , MIP-2, TNF- α , MCP-1, mKC, protein. Inhibition of HNE. LUNGS Histology and immunohistochemistry.
8	PBS	LPS	
4	Ad-lacZ	PBS	
8	Ad-lacZ	LPS	
4	Ad-elafin	PBS	
8	Ad-elafin	LPS	

Table 3. Experimental protocol used to determine the effect of Ad-elafin transfection on LPS-mediated airway neutrophilia.

On day 0 C57/Bl6 mice received an IT instillation (30µl) of PBS or Ad-lacZ (3x10⁷ pfu) or Ad-elafin (3x10⁷ pfu), n=12 in each group. Five days later, 8 mice in each group received IT LPS (0.5µg in 30µl) and the remaining 4 mice in each group received IT PBS (30µl). The following day mice were killed. Serum, BALF and lungs were retrieved.

A



B

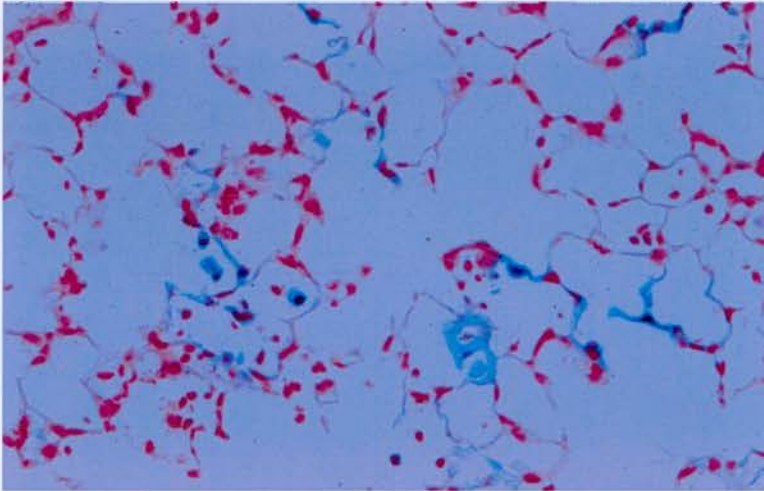


Figure 4. Intratracheal administration of adenovirus results in transfection of airway epithelium.

C57/Bl6 mice received an IT instillation of Ad-lacZ (3×10^7 pfu) followed five days later by an IT instillation of 0.5 μ g of LPS. Twenty-four hours later mice were killed. The lungs were retrieved and stained with X-gal solution. A blue colour indicates expression of transgene.

Panel A illustrates expression of transgene in bronchial epithelium (original magnification $\times 40$).

Panel B illustrates expression of transgene in alveolar epithelium. Alveolar macrophages are also stained (original magnification $\times 100$).

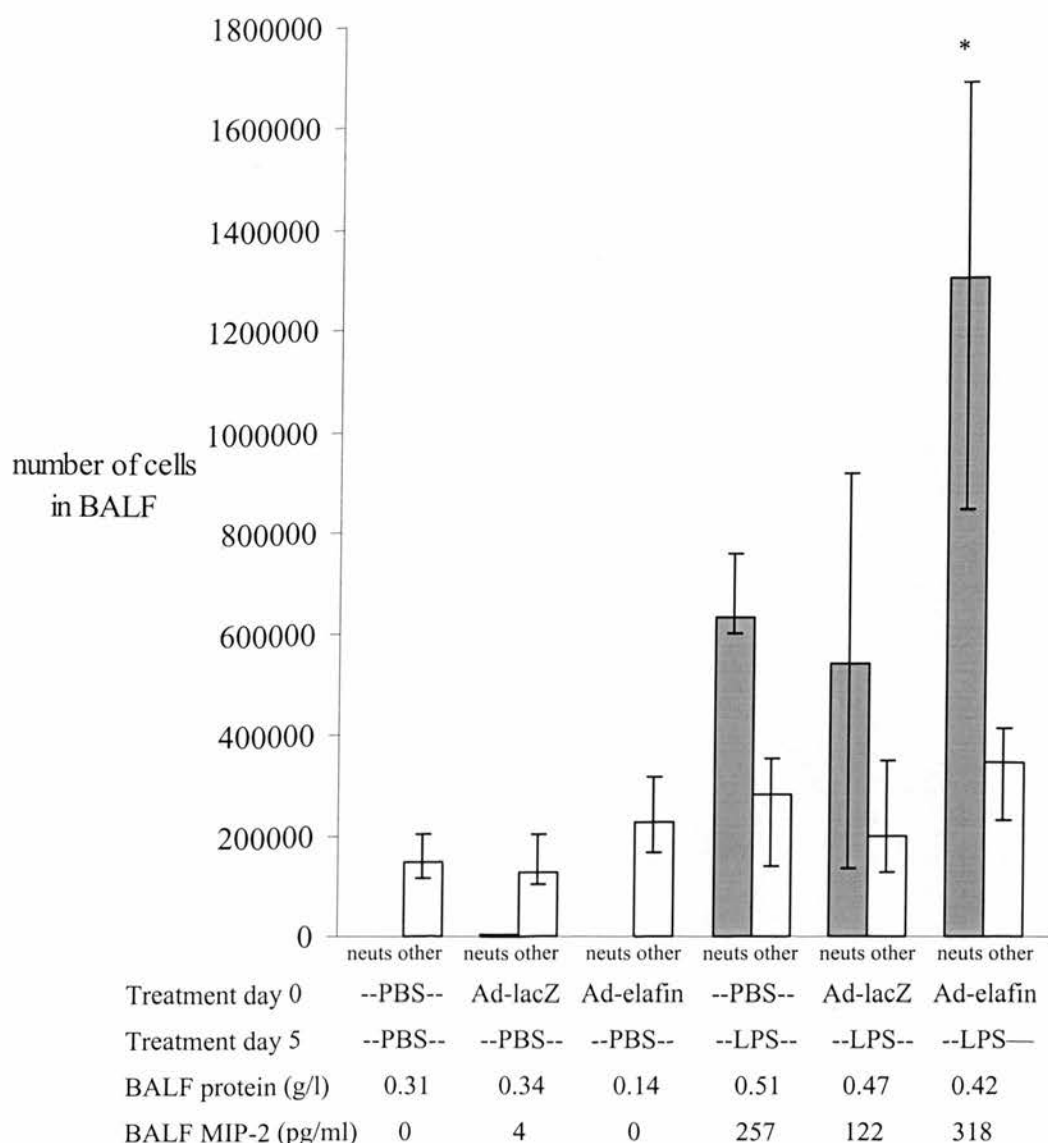


Figure 5. LPS-induced airway neutrophilia is enhanced by Ad-elafin.

As outlined in Table 3, C57/Bl6 mice received an IT instillation of Ad-elafin or Ad-lacZ (3×10^7 pfu) or PBS ($n=12$ in each group). Five days later 4 mice in each group received PBS IT, and the remaining 8 mice in each group received $0.5 \mu\text{g}$ LPS IT. Twenty-four hours later mice were killed and both serum and BALF retrieved. BALF total cell count, neutrophil count, protein concentration and MIP-2 concentration were measured.

Neutrophil count ('Neuts') in BALF is indicated by shaded boxes, and the remaining number of cells in BALF ('Other') by open boxes. Cell counts are presented as medians and interquartile ranges. Protein and MIP-2 concentrations are presented as medians.

* = significant difference in neutrophil count ($p < 0.05$) when compared with any of the other treatments.

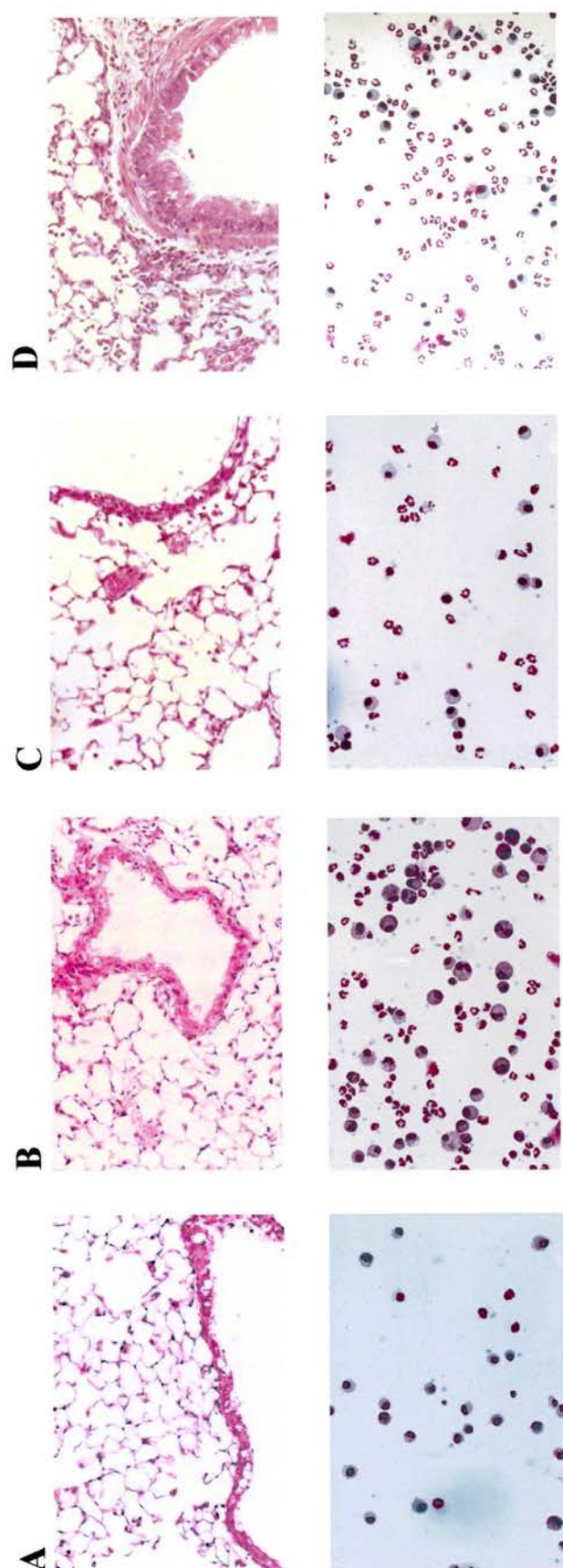


Figure 6. Ad-elafin treatment augments LPS-mediated pulmonary neutrophilia.

C57/Bl6 mice were treated as described in Table 3. BALF was performed and cytopins prepared. Lungs were retrieved, fixed, sectioned and stained with haematoxylin and eosin. Histology is represented in the upper panels and cytopins in the lower panels (original magnification x 100 in each case).

A: Representative appearances in mouse treated with Ad-elafin/PBS (almost identical patterns were observed in mice receiving PBS/PBS or Ad-LacZ/PBS).

B: Representative appearances in mouse treated with PBS/LPS.

C: Representative appearances in mouse treated with Ad-LacZ/LPS.

D: Representative appearances in mouse treated with Ad-elafin/LPS.

The concentration of the neutrophil chemokine MIP-2 in BALF from mice treated with LPS broadly followed the pattern of airway neutrophilia (ie highest levels in Ad-elafin/LPS mice, intermediate levels in PBS/LPS mice and lowest levels in Ad-lacZ/LPS mice) (Figure 5). However the correlation between MIP-2 and elafin concentrations in BALF from Ad-elafin/LPS mice was not statistically significant ($r=0.39$, $p=0.36$). Insufficient BALF was available to quantify other chemokines from individual mice. Instead equal volumes of BALF from the mice in each group described in Figure 5 were pooled for analysis of TNF- α , MIP-1 α , MCP-1 and mKC levels.⁴ With the limitations of pooled samples in mind it was nonetheless interesting that TNF- α and MIP-1 α concentrations followed the same pattern as described for airway neutrophilia and MIP-2 (ie highest levels in Ad-elafin/LPS mice, intermediate levels in PBS/LPS mice and lowest levels in Ad-lacZ/LPS mice). In contrast no mKC was detected in BALF from any of the groups, while MCP-1 was higher in PBS/LPS mice than in Ad-elafin/LPS mice than in Ad-lacZ/LPS mice.

Several additional points are made in Figure 5. For example, the cellular composition of BALF from PBS/PBS mice was almost identical to that of BALF from Ad-lacZ/PBS and Ad-elafin/PBS mice. The total cell count in BALF from Ad-elafin/PBS mice was a little higher than that from the other two groups, but remained well within the limits considered normal for BALF cell counts (see section 4.2.1.). It appears therefore that overt vector-induced neutrophilia did not occur in this experiment. This is supported by the cytopins and sections in Figure 6, in which BALF and lungs from Ad-elafin/PBS mice are seen to be virtually devoid of neutrophils (an identical picture was observed in PBS/PBS mice and Ad-lacZ/PBS mice, data not shown). Extending this theme, the observation that the protein concentration in BALF was similar in Ad-lacZ (or Ad-elafin)/PBS mice and in PBS/PBS mice implies that viral infection at the doses used did not compromise integrity of the alveolar-capillary membrane.

Under these circumstances it is extremely unlikely that Ad-elafin's effect on LPS-induced neutrophilia could be attributable to adenovirus *per se*, as virus was neither associated with neutrophilia nor epithelial leak. This is further supported by the observation that Ad-lacZ/LPS treatment was associated, if anything, with a small fall in neutrophil count relative to that in PBS/LPS mice (Figure 5).

⁴ I performed the MIP-2 ELISAs described in this work. However TNF- α ELISA was performed by Dr Jean-Michel Sallenave, and ELISAs for MIP-1 α , MCP-1 and mKC were performed by Professor T. Standiford, University of Michigan, Ann Arbor, USA.

In seeking potential explanations for the effect of Ad-elafin in augmenting LPS-induced neutrophilia, it is useful to consider BALF elafin levels (Figure 7). Elafin was not detected in BALF from any of the 24 mice which did not receive Ad-elafin. In contrast elafin was found in low concentration in BALF from Ad-elafin/PBS mice, with a significant (20-fold) up-regulation in Ad-elafin/LPS mice (Figure 7). There are three immediate implications arising from this observation.

Firstly, as the anti-elafin antibody used in the elafin ELISA does not recognise murine proteins, and as the Ad-elafin construct does not contain a 5' untranslated region, it appears that LPS (either directly or indirectly) stimulates the mCMV promoter to drive expression of human elafin. Further support for this phenomenon was provided by experiments in which mice were treated IT with Ad-lacZ (3×10^7 pfu), followed 5 days later by either 0.5 μ g or 5 μ g of LPS IT. Lungs were retrieved 24 hours later and stained for β -galactosidase (the product of the lacZ gene). LPS resulted in a dose-dependent increase in expression of β -galactosidase which in turn is under the control of the mCMV promoter (Figure 8).

Secondly, it might intuitively be supposed that augmented levels of elafin may influence neutrophil migration by an effect on murine elastase. Certainly human elafin is capable of inhibiting murine elastase *in vitro* (personal communication from Professor G. Lungarella, University of Siena, to Dr Jean-Michel Sallenave). The capacity of BALF to inhibit elastase was measured in a crude way, owing to limitations in the volume of BALF available from each mouse. 10 μ l of BALF was simply incubated with 10 μ l (100ng) of HNE for 30 minutes at 37°C before application of methoxysuccinyl-ala-ala-pro-val p-nitroanilide. The mean inhibition of HNE for each group was as follows: PBS/PBS, 75%; Ad-lacZ/PBS, 80%; Ad-elafin/PBS, 75%; PBS/LPS, 84%, Ad-lacZ/LPS, 81%, Ad-elafin/LPS, 79%. Whilst recognising the necessarily crude nature of this experiment it appears that no striking augmentation of elastase inhibitors in BALF occurred after Ad-elafin transfection.

Thirdly, it could potentially be argued that elafin influences neutrophil migration through a direct or indirect concentration-dependent chemotactic function. However neutrophil counts were lower in Ad-elafin/PBS mice than in Ad-lacZ (or PBS)/PBS mice. Similarly the correlation between elafin concentration and neutrophil count in Ad-elafin/LPS mice was not statistically significant. Therefore, while a direct effect of high concentrations of elafin on neutrophil migration cannot be categorically dispelled on the basis of the evidence available, it seems unlikely to

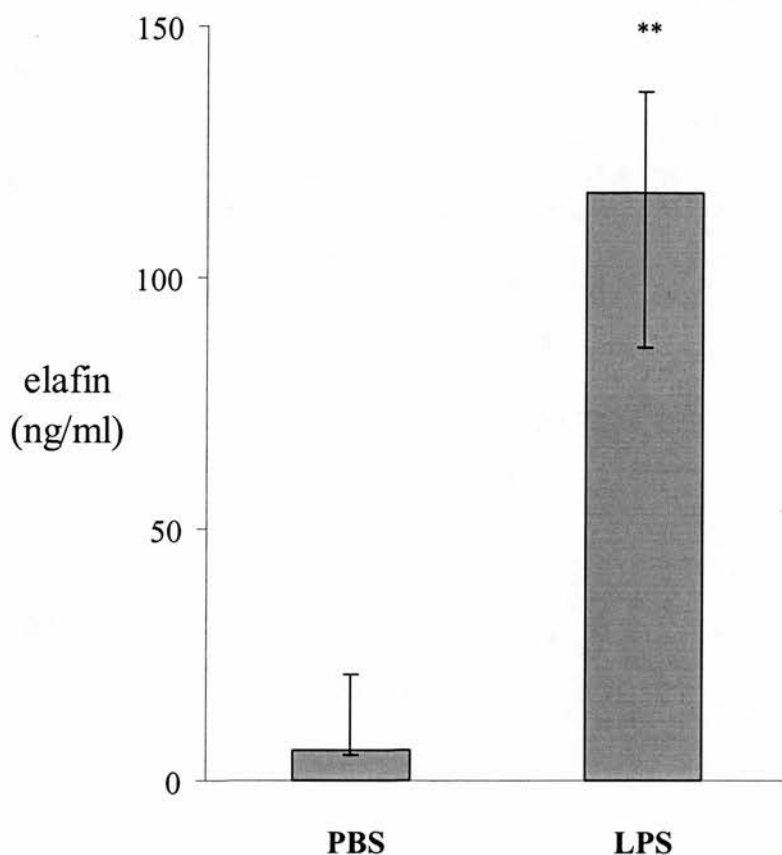


Figure 7. LPS significantly enhances elafin expression in Ad-elafin treated mice.

C57/Bl6 mice were treated IT with Ad-elafin (3×10^7 pfu) followed five days later by PBS (n=4) or 0.5 μ g LPS (n=8), as described in Table 3. BALF was retrieved 24 hours later and the concentration of human elafin determined by ELISA.

Results represent medians and interquartile ranges.

** = significant difference, $p < 0.01$, when comparing the two groups.

A**B**

Figure 8. LPS stimulates expression driven by the mCMV promoter *in vivo*.

C57/Bl6 mice were treated IT with Ad-lacZ (3×10^7 pfu) followed five days later by either 0.5 or 5 μ g of LPS. Twenty-four hours later mice were killed. Lungs were removed and stained with X-gal solution. Expression of transgene is indicated by blue colour.

PANEL A: Appearance of lungs stained after Ad-lacZ then 0.5 μ g LPS.

PANEL B: Appearance of lungs stained after Ad-lacZ then 5 μ g LPS.

explain the observations in Figure 5.

The observation of markedly elevated human elafin concentrations in BALF from mice treated with Ad-elafin then LPS prompted immunohistochemical studies aiming to determine the cellular sources of elafin after transfection (Figure 9).^{5 6} Surprisingly few cells were seen to stain positively for elafin antigen. Those that did included alveolar epithelial cells (Figure 9, Panels A and B) as well as inflammatory cells in alveolar spaces (Figure 9, Panels C and D). One striking feature was the detection of elafin on the brush border of ciliated bronchial epithelium (Figure 9, Panel E). Whilst staining of luminal interfaces is a recognised artefactual effect in immunohistochemistry, the fact that control sections did not reveal similar staining (Figure 9, Panel H) suggests that elafin does indeed concentrate on or around cilia, possibly reflecting high levels of secretion.

To summarise this section, low dose Ad-elafin administration generated human elafin in the murine airway without producing overt vector-induced inflammation. Ad-elafin was associated with a significant augmentation of LPS-induced pulmonary neutrophilia, and LPS induced a significant up-regulation of elafin transgene expression in murine airways.

4.2.6. The effect of Ad-elafin transfection on human neutrophil migration *in vitro*.

Transfection of A549 cells with Ad-elafin resulted in an increase in migration of human neutrophils across a polycarbonate filter of 3µm pore size (Figure 10). A viral effect appeared to contribute to the enhanced neutrophil migration, as Ad-lacZ transfection was consistently associated with higher neutrophil migration than was observed for untransfected cells (Figure 10), the difference approaching but not reaching statistical significance.

To confirm whether neutrophils were responsive to chemotactic stimuli in this system two wells containing untransfected A549 cells were prepared. Immediately prior to neutrophil-containing inserts being placed into the wells, fMLP (final concentration 10^{-8} M) was added to the insert being placed in the first well, and

⁵ Immunohistochemical staining was performed by myself and Mr Koen Schepers, a visiting student from the University of Leiden, who I helped to supervise during his attachment to Dr Sallenave's lab.

⁶ Slides of psoriatic skin (as positive control) were kindly provided by Dr Liz Sabin, Department of Pathology, University of Edinburgh.

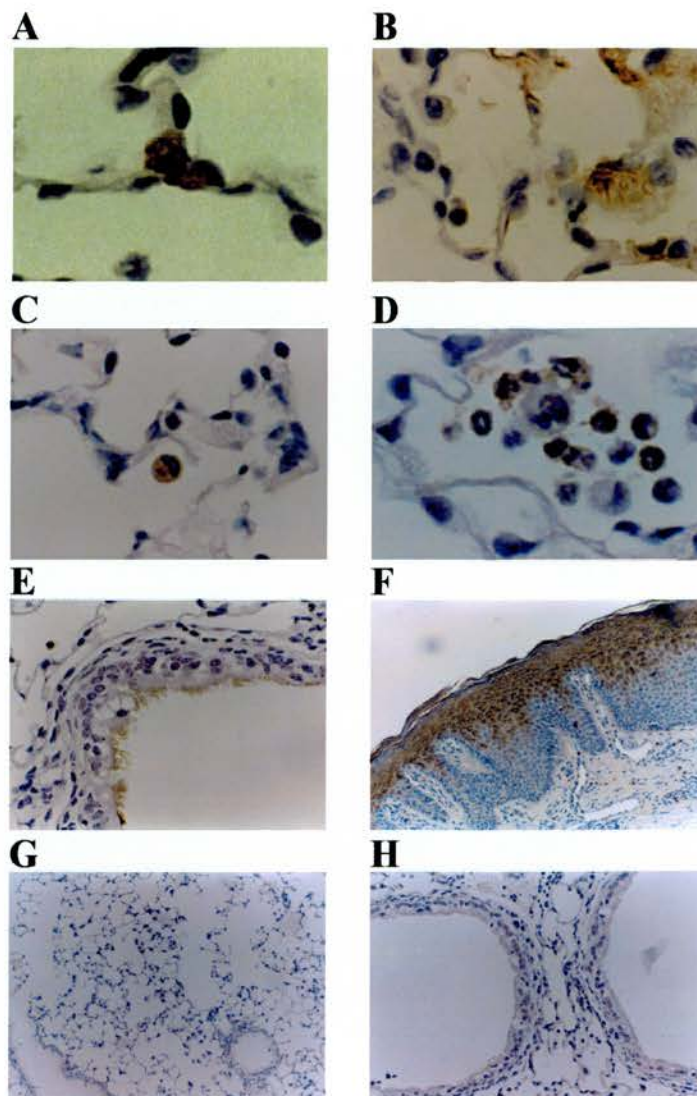


Figure 9. Immunohistochemical localisation of elafin after Ad-elafin transfection.

C57/Bl6 mice were treated as described in Table 3. Lungs were removed, fixed and sectioned. The presence of human elafin antigen was determined by immunohistochemistry using rabbit anti-human elafin immunoglobulin as primary antibody (PBS and pre-immune rabbit serum were used as controls). Secondary antibody comprised goat anti-rabbit immunoglobulin complexed to peroxidase. Upon addition of peroxidase substrate, positive staining is indicated by brown colour.

PANELS A-E: Ad-elafin/LPS mice, showing staining in alveolar epithelium

(A (original magnification x 400) and B (original magnification x 200)), inflammatory cells (C (original magnification x 200) and D (original magnification x 200)) and bronchial epithelial cilia (E (original magnification x 100)).

PANEL F: Positive control (psoriasis in human skin; original magnification x 100).

PANELS G AND H: Negative controls - Ad-lacZ/LPS mouse stained with anti-elafin antibody (G (original magnification x 40)), and Ad-elafin/LPS mouse stained with pre-immune serum (H (original magnification x 40)).

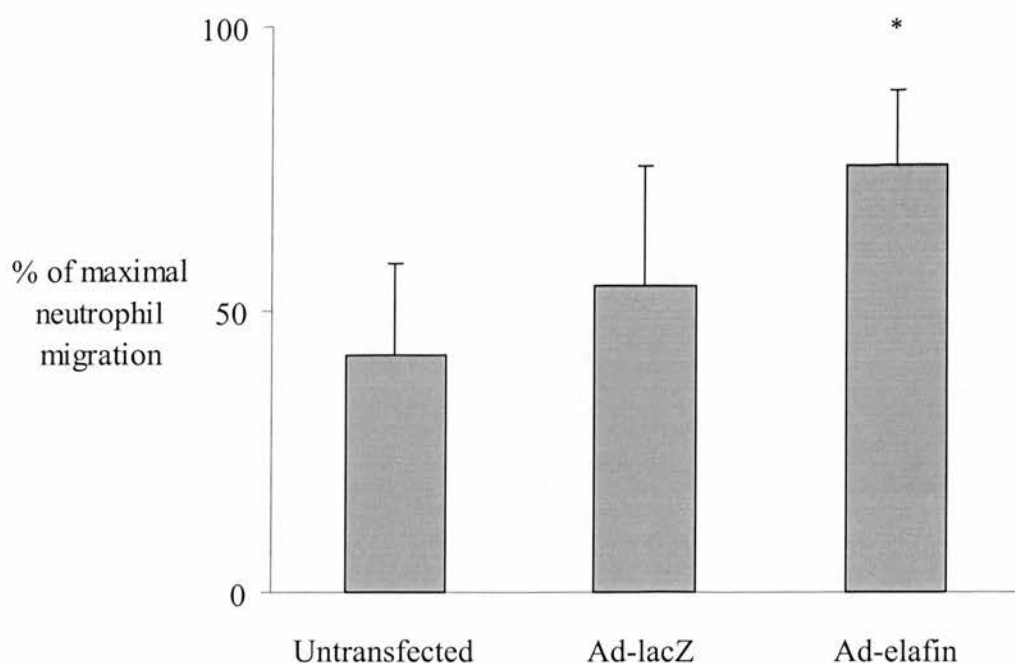


Figure 10. Ad-elafin transfection enhances neutrophil migration *in vitro*.

A549 cells were transfected with Ad-elafin or Ad-lacZ (moi of 50 for each) or vehicle alone and incubated in serum-free medium for 48 hours. 1.25×10^6 freshly isolated human neutrophils were applied to inserts housing polycarbonate filters of $3 \mu\text{m}$ pore size. The inserts were immediately placed in wells containing the A549 cells such that the bottom of each insert was in contact with conditioned medium, and incubated for 20 minutes at 37°C . Inserts were removed and discarded. Conditioned medium was treated with Triton X-100 to lyse migrated neutrophils prior to the addition of Na acetate-citrate buffer, pH 4.2. MPO activity was measured as a marker of neutrophil migration. Neutrophils from a single donor were used in each experiment, and each condition was performed in quadruplicate. In each experiment results were expressed relative to the well in which maximal neutrophil migration was observed.

Results are presented as means and standard deviations from 4 separate experiments.

* = significant difference when comparing Ad-elafin treatment with either of the other conditions, $p < 0.05$.

to the A549 cells of the second well (ie the first well had a potential chemotactic gradient favouring retention of neutrophils in the insert, while the second had a gradient favouring migration). At the end of the incubation period, the ratio of neutrophils in the insert to the number migrated was 5.9:1 in the first well and 1.8:1 in the second well. As such, neutrophils in this system were appropriately responsive to chemotactic stimuli.

Furthermore, in a separate experiment, neutrophil-containing inserts were incubated with Ad-elafin transfected A549 cells, and the elafin concentration in each compartment measured at the end of the experiment. The concentration of elafin in A549 cell conditioned medium was 95 ng/ml while the concentration in inserts was 17 ng/ml. This indicates that, while some elafin did permeate through the filter, a clear elafin gradient persisted throughout the incubation period. The demonstration of neutrophil responsiveness to chemokines and of preservation of potential chemotactic gradients in this experimental system suggest that the effects observed in Figure 10 were not non-specific.

Further experiments were performed in an attempt to identify factors influencing the observed increase in neutrophil migration associated with Ad-elafin. In 2 separate experiments, an excess of anti-elafin antibody was incubated with A549 cells prior to the addition of inserts. This resulted in loss of the effect of Ad-elafin over and above that attributable to Ad-lacZ (ie in the presence of antibody, mean % of maximal neutrophil migration was 64% for Ad-elafin transfected cells, 61% for Ad-lacZ treated cells, and 29% for untransfected cells). This suggested that the difference in neutrophil migration when comparing Ad-lacZ treatment and Ad-elafin treatment was indeed due to elafin.

Interestingly however, in a further experiment, addition of recombinant human elafin (final concentrations 50 and 100 ng/ml) to washed untransfected cells did not augment neutrophil migration (% of maximal neutrophil migration 46% for untransfected cells, 53% for untransfected cells incubated with 50 ng/ml elafin, 46% for untransfected cells incubated with 100 ng/ml elafin; for comparison the % of maximal migration for Ad-elafin transfected cells in the same experiment was 76%). Similarly, adding recombinant human elafin (final concentration 10^{-7} M to 10^{-4} M) to wells in the absence of A549 cells had no effect on neutrophil migration (% of maximal neutrophil migration 1% for medium alone, and 1%, 1%, 0% and 1% for elafin at 10^{-7} M, 10^{-6} M, 10^{-5} M and 10^{-4} M respectively). It appears therefore that

neutrophil migration in this system is induced by A549 cells, the effect being enhanced by Ad-elafin transfection.

The lack of effect attributable to recombinant elafin in this system was consistent with further studies in which recombinant elafin was not found to have direct neutrophil chemotactic activity in a cell-free chemotaxis assay.⁷ Interpretation of these data should however take into account the fact that recombinant elafin used in these experiments is a truncated molecule with much of the NH₂-terminal end of full-length elafin unrepresented.

On the basis that Ad-elafin transfection may induce generation of a chemotactic factor, levels of IL-8 were measured in conditioned media after incubation with neutrophil-containing inserts (Figure 11). Higher levels of expression of IL-8 from untransfected cells than from Ad-elafin transfected cells suggested that induction of IL-8 was not the mechanism responsible for the effect of Ad-elafin described in Figure 10. Pre-incubation of A549 cells with an excess of anti-human-IL-8 had little effect on neutrophil migration in this system (11%, 0% and 7% reduction in maximal neutrophil migration associated with untransfected cells, Ad-lacZ treated cells, and Ad-elafin treated cells, respectively).

4.2.7. The effect of LPS on elafin secretion from transfected and untransfected A549 cells.

The demonstration of LPS-inducible secretion of elafin after Ad-elafin infection of murine airways (section 4.2.5.) led to the suggestion that targeted transfection of human cell lines may also allow for inflammation-specific transgene expression. Given that alveolar epithelial cells and alveolar macrophages are logical targets for gene therapy strategies aimed at the distal airway, A549 cells and macrophages were identified as cells in which to pursue such a hypothesis.

In untransfected A549 cells LPS stimulated a dose-dependent increase in elafin secretion above the levels produced constitutively (Figure 12). Transfection with Ad-lacZ at an moi of 50 appeared to lead to a slight dampening of the response to LPS, particularly at lower doses (0.1-1 µg/ml) (Figure 12). In keeping with

⁷ The chemotaxis assay was kindly performed by Dr Marie-Hélène Ruchaud-Sparagano, Rayne Laboratory, University of Edinburgh.

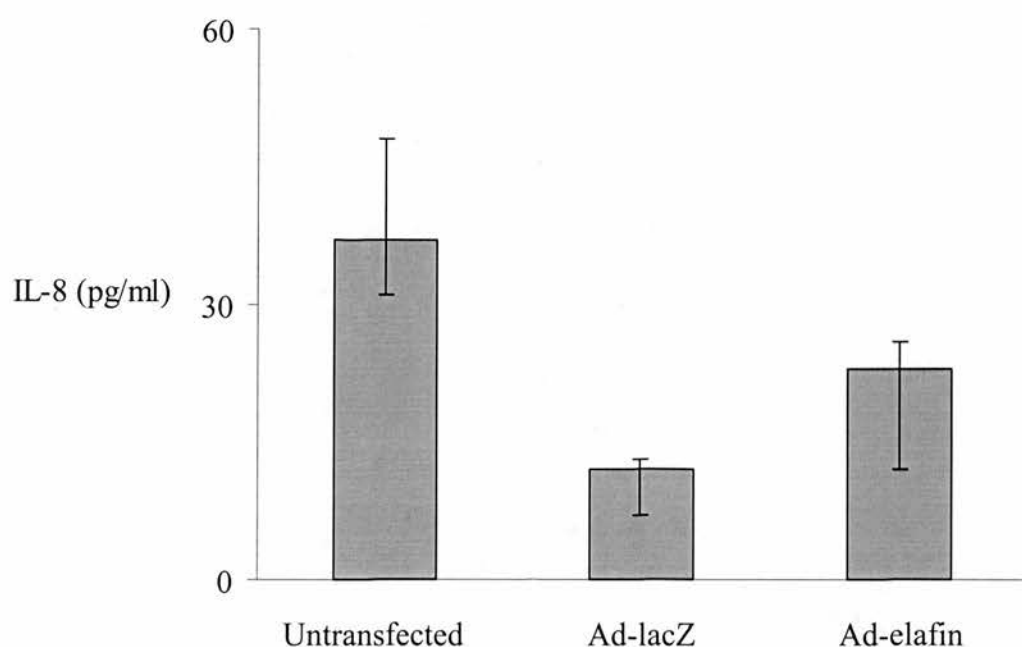


Figure 11. Increased neutrophil migration induced by Ad-elafin transfection of A549 cells is not associated with augmented IL-8 secretion.

A549 cells were treated with Ad-elafin or Ad-lacZ (moi of 50 in each case) or vehicle alone prior to the addition of inserts containing 1.25×10^6 neutrophils, using the protocol outlined in Figure 10. IL-8 concentrations in conditioned media were measured by ELISA.

Results represent medians and interquartile ranges derived from 3 separate experiments. No significant difference was found when comparing the three groups, $p=0.05$.

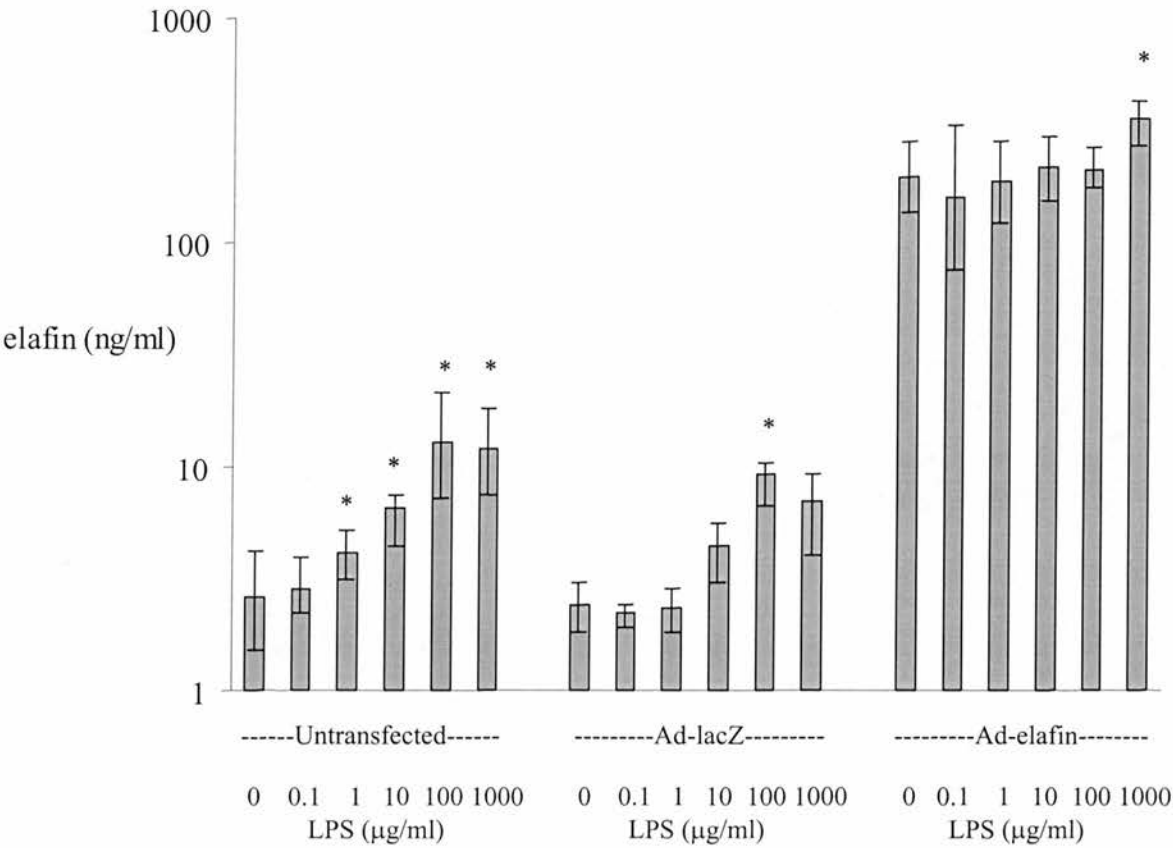


Figure 12. LPS augments elafin secretion from untransfected A549 cells, but only high concentrations of LPS increase elafin secretion from cells transfected with Ad-elafin at an moi of 50.

A549 cells were treated with Ad-elafin or Ad-lacZ (each at 50 moi) or vehicle alone and incubated in serum-containing medium for 48 hours. LPS was added over the dose range 0-1000 µg/ml for 24 hours, and elafin concentration was measured in supernatants by ELISA.

Data represent medians and interquartile ranges illustrated on a logarithmic scale, n=5. * = significant difference, $p < 0.05$ when compared with the baseline value (ie where no LPS added) for each treatment. In addition, for each of the concentrations of LPS applied, elafin levels were higher in supernatants from Ad-elafin treated cells than in supernatants from untransfected or Ad-lacZ treated cells ($p < 0.05$ in each case).

findings in Chapter 3, Ad-elafin transfection of A549 cells at an moi of 50 resulted in high levels of elafin secretion even in the absence of LPS (Figure 12). LPS generally had little effect on this high baseline level of elafin production, except at extremely high concentrations (1mg/ml) (Figure 12). To determine whether the apparent lack of LPS-responsiveness of transfected cells relative to untransfected cells was due to increased susceptibility of transfected cells to LPS toxicity, cell viability was measured at the conclusion of a representative experiment. For each treatment cell viability in monolayers approximated closely to that described in section 3.2.1., with no obvious toxic effect of LPS observed (data not shown).

On balance it was felt unlikely that LPS toxicity was responsible for the relative LPS-unresponsiveness of cells treated with Ad-elafin at an moi of 50. Instead it was considered possible that the powerful mCMV promoter was stimulating near maximal expression of elafin transgene in A549 cells at baseline, leaving little capacity for significant increase upon addition of LPS. Therefore, the experiments were repeated using adenoviral transfection at an moi of 1 (Figure 13).

Despite the reduced dose of adenovirus, Ad-lacZ transfection was again associated with a relative blunting of the characteristic effect of LPS on untransfected cells (Figure 13). In contrast LPS stimulation of Ad-elafin transfected cells resulted in a dose-dependent increase in elafin secretion (Figure 13). The magnitude of the LPS-induced rise in elafin secretion was higher in Ad-elafin treated cells than in untransfected (or Ad-lacZ treated) cells, and it is assumed that the increment is attributable to elafin expression driven by the mCMV promoter.

4.2.8. The effect of LPS on elafin secretion from transfected and untransfected monocyte-derived macrophages.

Adenoviral transfection of monocyte-derived macrophages proved to be feasible, with a transfection efficiency calculated at approximately 16% (Figure 14). Experiments were therefore carried out to determine whether LPS could up-regulate elafin secretion from macrophages (Figure 15). It must be emphasised that these experiments were performed using autologous human serum (as a source of LPS binding protein), and that human serum is known to contain elafin (Molhuizen and Schalkwijk, 1995). In this study the concentration of elafin in medium containing 10% autologous human serum was approximately 1.7 ng/ml. It is immediately apparent from Figure 15 that levels of elafin in medium obtained from untransfected

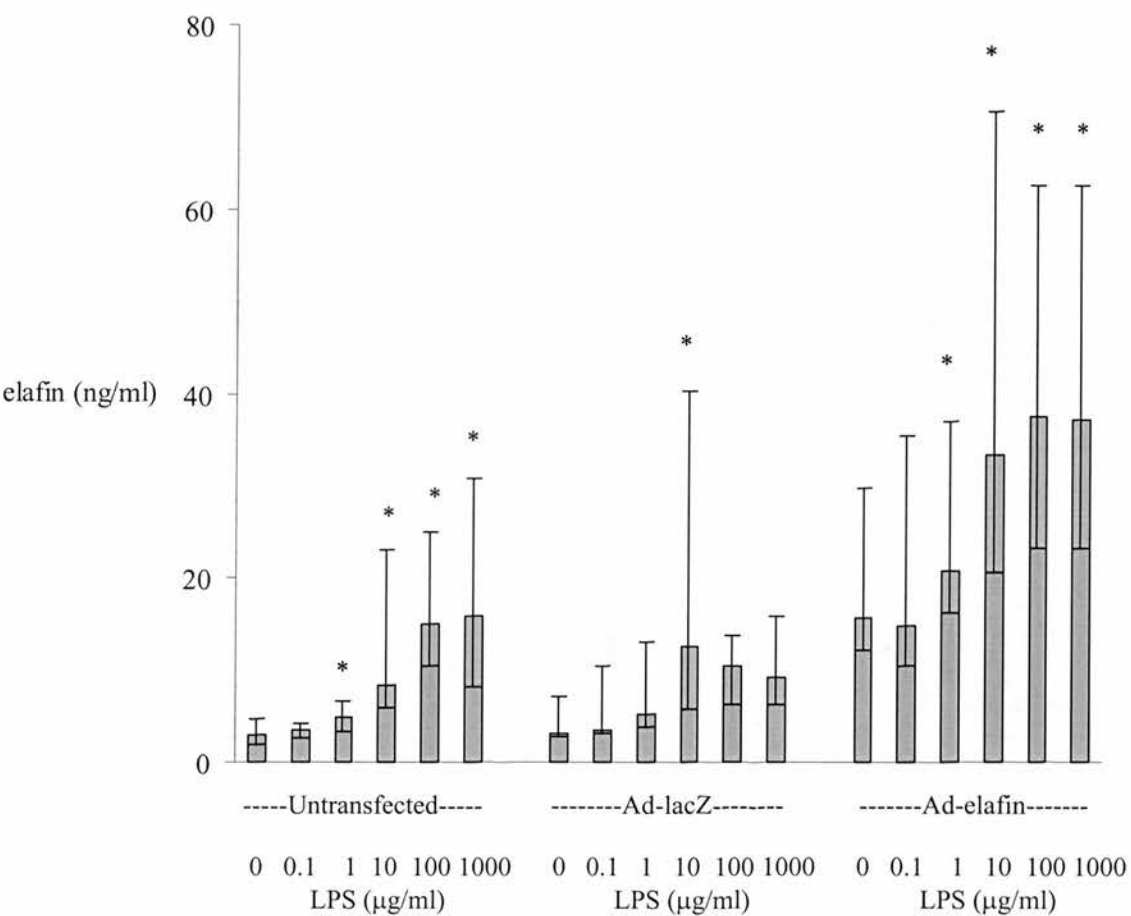


Figure 13. LPS up-regulates elafin secretion from A549 cells transfected with Ad-elafin at an moi of 1.

A549 cells were treated with Ad-elafin or Ad-lacZ (each at 1 moi) or vehicle alone and incubated in serum-containing medium for 48 hours. LPS was added over the dose range 0-1000 µg/ml for 24 hours, and elafin concentration was measured in supernatants by ELISA.

Data represent medians and interquartile ranges, n=5.

* = significant difference, $p < 0.05$ when compared with the baseline value (ie where no LPS added) for each treatment. In addition, for each of the concentrations of LPS applied, elafin levels were higher in supernatants from Ad-elafin treated cells than in supernatants from untransfected or Ad-lacZ treated cells ($p < 0.05$ in each case).

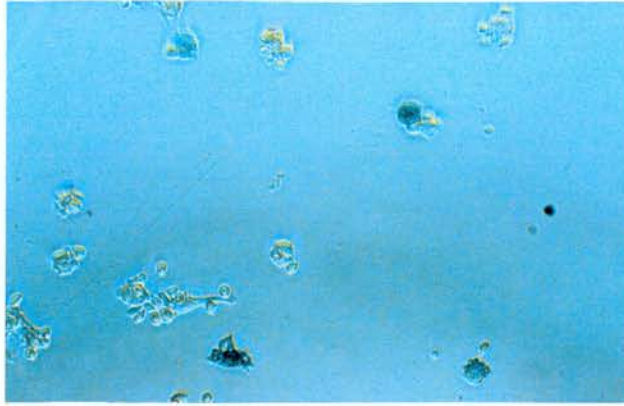
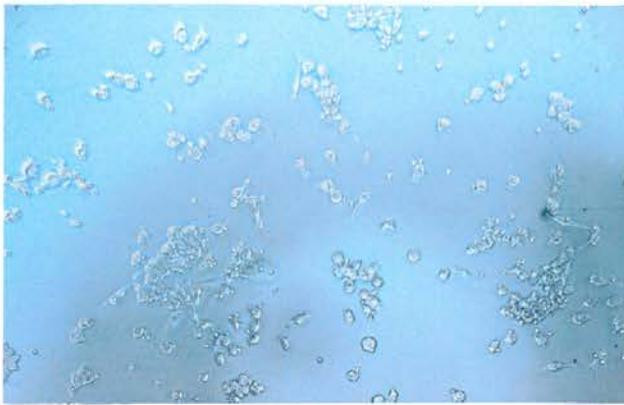
A**B**

Figure 14. Transfection of monocyte-derived macrophages with Ad-lacZ.

Monocyte-derived macrophages were treated with Ad-lacZ (PANEL A; moi of 50 - original magnification x 100) or vehicle alone (PANEL B - original magnification x 100) and incubated in serum-containing Iscove's Medium for 48 hours. Cells were then treated with X-gal stain. Blue colouration indicates expression of the β -galactosidase gene.

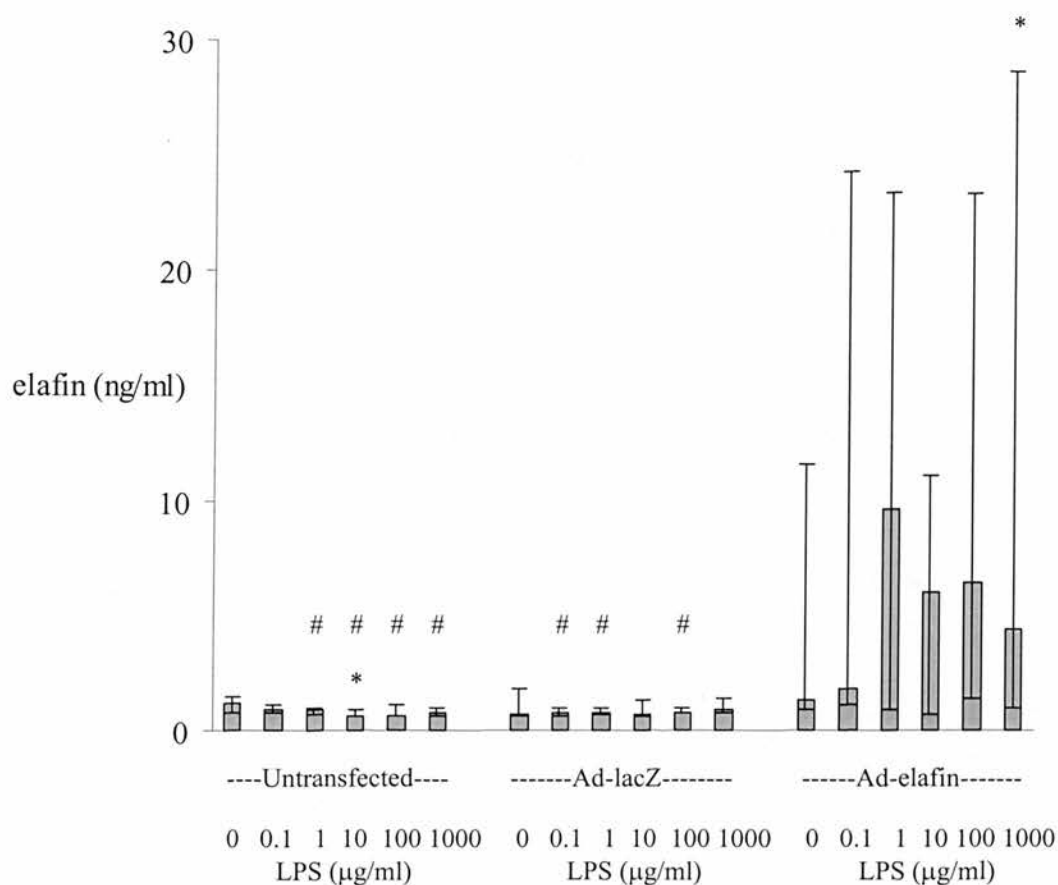


Figure 15. LPS up-regulates elafin production from Ad-elafin transfected monocyte-derived macrophages.

Monocyte-derived macrophages were treated with Ad-elafin or Ad-lacZ (each at 50 moi) or vehicle alone and incubated in serum-containing medium for 48 hours. LPS was added over the dose range 0-1000 µg/ml for 24 hours, and elafin concentration was measured in supernatants by ELISA.

Data represent medians and interquartile ranges, n=6.

* = significant difference, $p < 0.05$ when compared with the baseline value (ie where no LPS added) for each treatment. # = significant difference, $p < 0.05$ compared with elafin levels from Ad-elafin treated cells receiving the equivalent dose of LPS.

and Ad-lacZ treated macrophages exposed to LPS approximated to the level attributable to human serum. Intriguingly, there appeared to be a trend for LPS to reduce slightly the concentration of elafin in conditioned medium from untransfected macrophages, this becoming statistically significant at 10 μ g/ml LPS (Figure 15).

In contrast, Ad-elafin transfection was associated with elafin secretion which was stimulated by exposure to LPS, though this effect was only statistically significant when extremely high concentrations (1 mg/ml) of LPS were applied (Figure 15). One striking feature of Figure 15 is the size of the error bars, reflecting the considerable inter-individual variation in elafin secretion in response to LPS. When the data in Figure 15 were analysed donor by donor, it became apparent that macrophages from 3 donors readily secreted elafin in response to LPS, whilst no definite LPS-induced up-regulation of elafin secretion was observed in macrophages from the remaining 3 donors.

The apparent absence of elafin production in untransfected and Ad-lacZ transfected macrophages exposed to LPS raised three questions. Firstly, were the macrophages isolated intrinsically resistant to LPS? Secondly, did LPS induce macrophage death thereby precluding evidence of LPS-mediated up-regulation of elafin? Thirdly, are monocyte-derived macrophages capable of constitutively producing elafin?

Taking these questions in turn, IL-8 secretion was measured in a subset of macrophages exposed to LPS (Table 4). The characteristic LPS-dependent increase in IL-8 secretion in both transfected and untransfected macrophages indicated not only that the cells were LPS-responsive, but that adenoviral transfection did not obviously affect the response. With regard to the susceptibility of isolated macrophages to LPS toxicity, the significant majority of macrophages were adherent to plastic wells, and cell viability approximated to 100% irrespective of transfection or dose of LPS. It was therefore concluded that LPS (or viral) toxicity did not appear to explain the apparent lack of secretion of endogenous elafin in response to LPS.

This leaves the question of whether elafin can be produced constitutively by human monocyte-derived macrophages. Two strands of evidence suggest that macrophages are capable of producing elafin when exposed to LPS. Firstly, in 2 separate experiments the protocol described in Figure 15 was followed with the

	Dose of LPS (µg/ml)					
	0	0.1	1	10	100	1000
Untransfected	11.2	56.8	63.8	67.1	88.6	87.3
Ad-lacZ	7.5	46.8	56.7	65.8	89.7	95.5
Ad-elafin	11.9	46.3	58.0	63.7	86.7	87.7

Table 4. LPS treatment of monocyte-derived macrophages was associated with an appropriate rise in IL-8 secretion.

Monocyte-derived macrophages were treated with Ad-elafin or Ad-lacZ (each at 50 moi) or vehicle alone and incubated in serum-containing medium for 48 hours. LPS was added over the dose range 0-1000 µg/ml for 24 hours, and IL-8 concentration (shown in bold print and expressed in pg/ml) was measured in supernatants by ELISA. Data are derived from a single representative experiment.

exception that FCS replaced autologous human serum. In one of these experiments no elafin was detected in supernatants from untransfected or Ad-lacZ treated macrophages either in the absence or presence of LPS. A similar pattern emerged in the second experiment, with the exception of Ad-lacZ treated macrophages stimulated with LPS at 1 mg/ml, in which elafin was unequivocally detected at a concentration of 1.1 ng/ml. Secondly, Ad-lacZ transfected macrophages from 1 of the 6 donors contributing to the data in Figure 15 were associated with a small but unequivocal increase in elafin levels when comparing application of 1 µg/ml LPS and no LPS. In summary therefore, human monocyte-derived macrophages appear to be capable of generating elafin, but in this study this was only achieved in macrophages transfected with adenovirus and exposed to LPS; no elafin was detected in untransfected macrophages in the absence of LPS.

In this context the LPS-induced up-regulation of elafin secretion from Ad-elafin treated macrophages from some (but not all) donors (Figure 15) is very likely to result from the effect of LPS on the mCMV promoter. In 2 separate experiments, the timing of exposure to LPS was altered to coincide with the timing of adenoviral transfection (ie supernatants were retrieved 72 hours after Ad-elafin transfection with LPS applied throughout; in contrast, in Figure 15 supernatants were recovered 72 hours after transfection but LPS was applied only during the final 24 hours). Figure 16 demonstrates the striking up-regulation of elafin secretion with relatively low concentrations of LPS under these circumstances, reinforcing the point that Ad-elafin treated macrophages can potentially be stimulated into producing significant quantities of elafin.

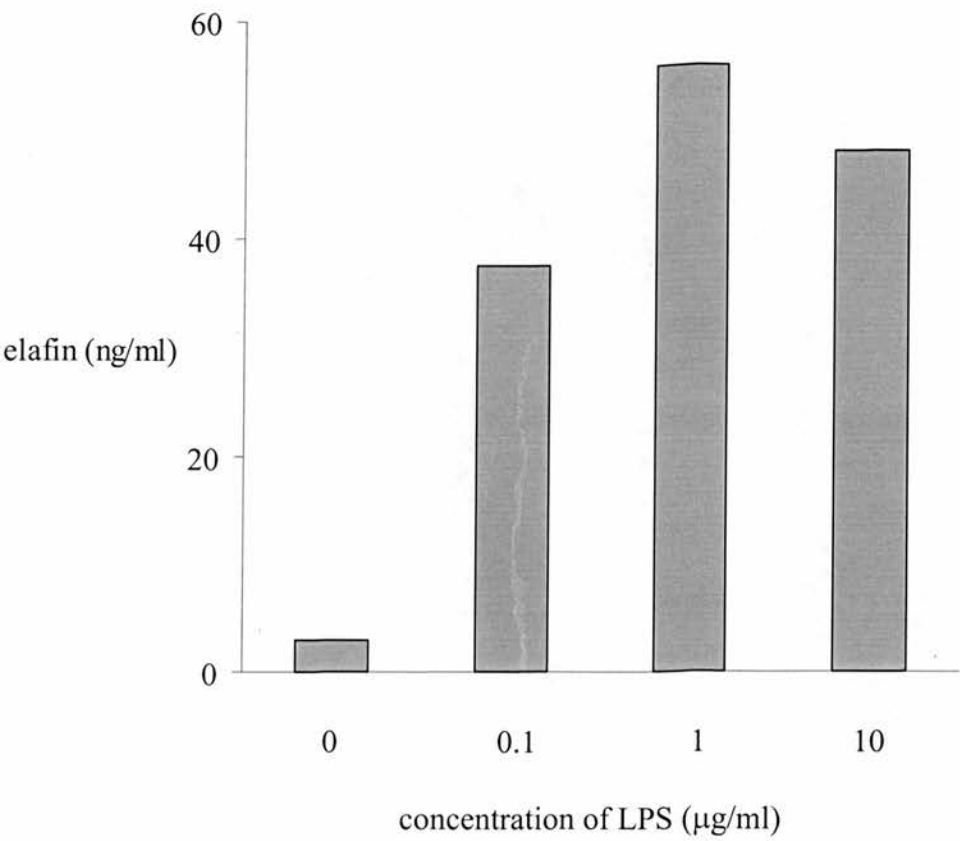


Figure 16. LPS-mediated up-regulation of elafin secretion from Ad-elafin transfected macrophages appears more efficient if LPS is applied at the time of transfection.

Monocyte-derived macrophages were treated with Ad-elafin (50 moi) and LPS (over the dose range 0-10 µg/ml) and incubated in serum-containing medium for 72 hours. Elafin concentration was measured in supernatants by ELISA.

Data represent means, n=2.

4.3. DISCUSSION

4.3.1. Technical considerations.

The principal conclusion arising from the data in this chapter is that Ad-elafin transfection of murine airways enhances LPS-induced neutrophilia. In interpreting these data a number of potential technical limitations should first be considered. These include factors relating to the route of administration used, the strain of mouse used, the control vector used, the structure of murine airways, and the anti-elastase properties of the murine lung.

4.3.1.1. Route of administration.

The variable distribution of vehicle associated with the IN route was disappointing, given that IN application is quick, simple, non-invasive and easily repeatable. The inherent variability is still more disappointing when it is considered that IN administration of Ad-elafin generated impressive elafin concentrations in the airways without obvious increases in BALF cell count (Table 1). The apparent lack of vector-induced inflammation should be interpreted cautiously however, in that IN administration of trypan blue resulted in almost exclusively apical deposition of dye. It is plausible therefore that there was indeed regional cellular influx (and high elafin expression) in the apices, but that BALF (which is presumed to mix fluids from all regions of the lung) dilutes out this effect. The IP route was similarly unsuitable to the requirements of this study in that serum levels of elafin were disappointingly low (Table 2).

In contrast, the IT route resulted in dispersion of vector to all regions of the lung, and consistently generated human elafin. The other principal advantage of the IT route over the IN route was the reassurance that all of the dose given entered the airways (in contrast to the variable gastrointestinal deposition associated with the nasal route). Despite these considerable advantages, the IT route is not without potential problems. For example, slightly deeper anaesthesia is required for this more invasive procedure and it is quite possible that a degree of airway trauma is induced by the small head of pressure used to instil vehicle. These problems were considered of negligible importance in this study as mice generally tolerated the procedure well, and mice in all groups were treated equally. Of potentially more concern was the variability in responsiveness of mice to the same IT stimulus. Using the example of

PBS/LPS mice in Figure 5, total cell count ranged from 120,000 to 1,364,000, though the percentage of neutrophils in BALF was less variable (55%-78% of total cell count). The observed variability is likely to represent either differences in the response of individual mice or variability in deposition of LPS in airways. In this context the relatively consistent percentage of neutrophils in the airway is reassuring, as the percentage was characteristic of good LPS exposure even in mice with relatively low total cell counts.

This theme becomes important in the context of ensuring equitable exposure to adenovirus in individual mice. Figure 1 provided valuable data in describing vector-induced neutrophilia associated with doses of adenovirus at 3×10^8 pfu and 1×10^9 pfu. However the higher total cell (and neutrophil) counts in mice receiving 3×10^8 pfu as compared with those receiving 1×10^9 pfu was unexpected. Again it seems likely either that deposition of virus was variable, or that mice have a markedly variable inflammatory response to overtly immunogenic doses of virus. While there may have been some variability in viral infection, there is little doubt that transfection was efficient using the IT route, as elafin was detected in BALF from 38 of the 39 Ad-elafin treated mice described in this chapter, and Ad-lacZ transfection proved efficient as assessed by X-gal staining (Figure 4), a system which probably under-represents efficiency of adenoviral transfection (Couffignal et al., 1997). It should be mentioned also that variability both in transgene expression and immune response is well recognised after IT delivery in rodent models, as exemplified by two important early gene therapy studies using doses of adenovirus similar to those described here (Rosenfeld et al., 1991; van Ginkel et al., 1995).

Importantly in the context of this study, the problem of variability was much less pronounced when adenovirus was applied at 3×10^7 pfu, as illustrated by consistent low level expression of elafin using Ad-elafin at this dose (Figure 1 and left hand column of Figure 7), and the consistently normal composition of BALF retrieved from Ad-elafin and Ad-lacZ treated mice not exposed to LPS (Figures 1 and 5). On the basis of the available evidence therefore, it seems that variability in the innate immune response to adenovirus becomes important above a certain dose threshold (in this case somewhere between 3×10^7 pfu and 3×10^8 pfu), but below this threshold overt vector induced inflammation is low or absent. Indeed the determination of a dose of adenovirus causing little inflammation in the airways whilst allowing transgene expression may have far reaching consequences for gene

therapy. This point is expanded upon later in this chapter when discussing the effect of LPS administration on transgene expression.

In summary, the IT route was safe and provided efficient and reproducible delivery of LPS and adenovirus to the airways. IT administration of adenovirus at low dose (3×10^7 pfu) was associated with efficient transgene expression with minimal variability in the total cell count (or neutrophil count) in BALF.

4.3.1.2. Strain of mouse used.

C57/Bl6 mice were used principally because this strain has been extensively studied in gene therapy protocols, and because of local experience with this strain as a background for the generation of elafin transgenic and CFTR "knockout" models (Dorin et al., 1992). Interestingly the C57/Bl6 mouse seems to be relatively tolerant of recombinant adenovirus, allowing prolonged heterologous transgene expression, particularly after intravenous administration (Barr et al., 1995; Michou et al., 1997). This is consistent with the expression of elafin in BALF for at least 8 days via the IT route (Figure 1) and at least 9 days via the IN route (Table 1), with no obvious extinction of signal when considering vector doses of 3×10^7 pfu. C57/Bl6 mice therefore appeared well suited to the development of models requiring transgene expression for a few days only.

4.3.1.3. Comparison of Ad-elafin and Ad-lacZ vectors.

Adenoviral vectors encoding the β -galactosidase gene from *E. coli*, or the luciferase fire-fly gene have become established as standard controls in the field of gene therapy. This largely reflects the fact that both can be usefully applied as "reporter" genes to provide a simple visual read out of transfection efficiency, as is demonstrated by staining for the presence of β -galactosidase at various points in this thesis. The considerable experience with these vectors has cemented their place as routinely used viral controls. However, potential criticisms of these vectors can be made. In the context of this work, a potential criticism is the use of a bacterial gene expressing an intracellular product (as in Ad-lacZ) with a human gene expressing a secreted product (as in Ad-elafin). The immune response to adenovirus (Yang et al., 1994; van Ginkel et al., 1995) has already been discussed in section 1.7.4. There is no doubt that murine immune responses are generated against bacterial and human gene products, and that the response to each is different (Kaplan et al., 1997; Michou

et al., 1997; Song et al., 1997a; McLachlan et al., 2000). In general human genes persist for longer than bacterial genes in C57/Bl6 mice (Michou et al., 1997; Song et al., 1997a). However evidence exists to suggest that the complete elimination of Ad-lacZ from the lungs of C57/Bl6 mice occurs after day 10 (Michou et al., 1997).

On balance, whilst the intrinsic differences between Ad-lacZ and Ad-elafin need to be considered, the available evidence suggests that these had little impact on the results in this chapter. In particular, the vast majority of the genetic sequences in the two vectors are viral and of identical composition (Addison et al., 1997; Sallenave et al., 1998). In addition, persistence of Ad-lacZ was readily demonstrated at 6 days in this study (Figure 4). Finally, using cellular content of BALF as a very crude index of immune response, no obvious differences were apparent when comparing Ad-lacZ/PBS and Ad-elafin/PBS mice (Figure 5).

4.3.1.4. The structure of murine airways, and the anti-elastase properties of murine lungs.

The murine lung differs considerably from the human lung. Principal differences include the predominance of Clara cells throughout murine airways (in contrast to their characteristic presence in respiratory bronchioles in humans), and the relative paucity of cells producing mucus (Pack et al., 1981). Ms. Bianca Hiemskerk has isolated murine Clara cells in this laboratory, and she and I have demonstrated their efficient transfection with adenovirus *in vitro* (data not shown).

With regard to murine anti-proteases, the predominant species in plasma are α_1 -PI and contrapsin (Takahara and Sinohara, 1983). In contrast to the situation in man, five structurally related α_1 -PI genes have been isolated in the mouse (Borriello and Krauter, 1991), but only the two species containing a methionine in the active site are capable of inhibiting HNE (Paterson and Moore, 1996). On the other hand, contrapsin has no activity against elastase (Takahara and Sinohara, 1983). In recent years murine SLPI has also been identified as a single copy gene in mice, and the gene product efficiently inhibits HNE (Zitnik et al., 1997). However, to date a murine homologue of elafin has not been convincingly identified. The data in this chapter confirm that BALF from PBS/PBS mice is capable of inhibiting HNE efficiently, and it is presumed that most if not all of the constitutive elastase inhibition is provided by α_1 -PI and/or SLPI. Importantly, if a murine homologue of elafin does exist it does not cross-react with the antibody in the elafin ELISA used

here, as elafin was not detectable in BALF from any of the control animals in this work.

4.3.2. Augmentation of LPS-induced neutrophilia by Ad-elafin.

The striking augmentation of LPS-induced neutrophilia by Ad-elafin (Figures 5 and 6) seems unlikely to have been influenced significantly by the factors discussed in the previous section. In particular, the available evidence implies that the bacterial gene in Ad-lacZ would, if anything, tend towards increased inflammation over the effect of the human transgene in Ad-elafin. Furthermore, any confounding effect of the inherently variable response of murine lungs to inflammatory stimuli was (at least to some degree) reduced by using a system in which delivery is directly visualised, and by using relatively high numbers of mice. Finally, *in vitro* evidence using genetic augmentation of elafin in human epithelial cells provided support for an effect of Ad-elafin in promoting neutrophil migration (Figure 10).

This section will therefore address the consequences of the pattern of elafin secretion in murine airways, and consider how elafin may potentially exert its effect on neutrophil recruitment.

4.3.2.1. The effect of LPS on expression of elafin in murine airways transfected with low dose Ad-elafin.

A potentially important finding described in this chapter is the 20-fold increase in elafin secretion induced by LPS in Ad-elafin treated mice (Figure 7). This observation strongly implies that the mCMV promoter is activated by LPS, a conclusion supported by the LPS-dependent increase in β -galactosidase expression in the lung (Figure 8). Interestingly LPS is similarly known to stimulate the human CMV promoter (Löser et al., 1998). The inducibility of the mCMV promoter by LPS is in keeping with the description of LPS-induced stimulation of promoters from murine acute phase protein genes such as complement factor 3 and serum amyloid A3 (Varley et al., 1995). The potential may therefore exist to design vectors for gene therapy protocols which are up-regulated specifically in response to local inflammation. While extremely elegant and well characterised inducible promoters such as the tetracycline- and ecdysone-inducible gene expression systems already

exist, these are necessarily dependent on administration of an exogenous "switch" (Gossen et al., 1995; Kistner et al., 1996; No et al., 1996).

The potential advantage of the mCMV promoter in this setting is its inherent strength (Sallenave et al., 1998). In this chapter the significant LPS-induced generation of elafin (Figure 7) was achieved using a vector dose low enough to avoid overt airway inflammation as assessed by neutrophil influx and BALF protein concentration (Figure 5). Indeed the dose of Ad-elafin used here is considerably lower than doses used in conventional IT gene therapy protocols. It appears therefore that appropriate selection of powerful, inflammation-specific promoters could allow significant transgene expression whilst avoiding vector-induced tissue injury.

Despite the marked induction of elafin secretion by LPS, surprisingly few cells stained positively for human elafin (Figure 9). Two potential explanations arise. Firstly, the antibody used may have been inefficient at detecting elafin in histological sections from mouse lung. Against this argument is the intense staining found in histological sections of skin from patients with psoriasis (Figure 9, Panel F), although it remains possible that staining is less efficient in murine than in human tissue. Alternatively, it may indeed be that small numbers of airway cells are transfected but that these produce significant quantities of elafin, which in turn are secreted. Elafin is known to contain a signal peptide and to be rapidly secreted (Sallenave et al., 1993). Furthermore, intracellular demonstration of elafin has been localised to secretory apparatus in keratinocytes (Pfundt et al., 1996). Similarly the observation that elafin appeared to coat brush borders may imply rapid secretion but slow clearance from the lung (Figure 9, Panel E). Interestingly, interstitial staining was also conspicuous by its absence, despite the potential for elafin to bind interstitial substrate (Schalkwijk et al., 1999). This could potentially be explained by the fact that tissue binding is mediated by sequences in the NH₂-terminal domain of elafin, which may potentially be cleaved from the COOH-terminus upon tissue binding (Schalkwijk et al., 1999) thus obviating recognition by the antibody used in experiments described here (which was raised against the COOH-terminus of elafin).

If it were indeed the case that Ad-elafin transfection was only required in a small number of cells in order to effect elafin expression this would consolidate the impression that mCMV containing vectors may be appropriate in the therapeutic setting.

4.3.2.2. Could Ad-elafin's effect on LPS-induced neutrophilia be mediated by a direct effect of human elafin?

The observation that the enhancement of LPS-induced neutrophilia by Ad-elafin transfection was associated with up-regulated elafin secretion (Figures 5 and 7) raised the possibility that elafin may exert a direct effect on neutrophil migration into the lung. Any such inference needs to be viewed in the light of evidence relating to the role of elastase and its inhibitors in neutrophil migration.

There is a considerable body of evidence suggesting a role for neutrophil elastase in neutrophil migration. In the context of this study, LPS has previously been shown to up-regulate expression of active, surface bound HNE on neutrophils (Owen et al., 1995). HNE stimulates the expression of CD18 and CD11b by neutrophils (Woodman et al., 1993) and furthermore appears to be an endogenous ligand for the CD11b/CD18 integrin complex thereby affecting neutrophil adhesion by competing with alternative ligands such as fibronectin, C3bi and ICAM-1 (Cai and Wright, 1996). HNE is capable of increasing epithelial cell permeability (Peterson et al., 1995), and can generate chemotactic fragments from the fibrin degradation products resulting from inflammatory processes (Leavell et al., 1996). Conversely, HNE may potentially reduce chemotactic potential by cleaving IL-8 (Leavell et al., 1997).

The relationship between elastase and neutrophil migration becomes considerably more complex when considering the role of elastase inhibitors.

In the elegant intravital microscopy studies performed by Woodman et al. (1993), synthetic elastase inhibitor inhibited neutrophil adhesion to, and migration across, vessels in the rat mesentery. A separate group suggested that elastase inhibition by a synthetic compound reduced neutrophil migration *in vitro* by inhibiting the activation of gelatinase B (Delclaux et al., 1996).

In support of an inhibitory role for anti-elastases, α_1 -PI was shown to inhibit HNE-induced epithelial permeability (Peterson et al., 1995), to inhibit neutrophil migration in response to fMLP (Stockley et al., 1990), and to neutralise partially the chemotactic activity of bronchial epithelial cells induced by LPS (Koyama et al., 1995). However, other studies have suggested that the concentration and conformation of α_1 -PI may dynamically alter the molecule's effect on neutrophil

migration. Thus low concentrations of α_1 -PI were found to stimulate chemotaxis and chemokinesis, whilst higher concentrations had an inhibitory effect (Aoshiba et al., 1993). Furthermore oxidised α_1 -PI and low molecular weight cleavage products of α_1 -PI have been shown to increase neutrophil motility (Banda et al., 1988; Stockley et al., 1990), as have elastase- α_1 -PI complexes (Joslin et al., 1992).

SLPI has been shown to have mild inhibitory effects on fMLP-stimulated neutrophil chemotaxis (Stockley et al., 1990), and to have no effect on migration across primary human pulmonary vascular endothelial cells (Mackarel et al., 1999).

It should be noted that all of the studies cited in relation to neutrophil migration in this section describe *in vitro* experiments, with the exception of the work by Woodman et al. (1993). The recurring theme in animal studies is for SLPI or elafin to exert an inhibitory effect on neutrophil extravasation. For example truncated recombinant SLPI inhibited the pulmonary neutrophilia associated with inhaled HNE in guinea pigs (Suzuki et al., 1996), while anti-SLPI antibody administered IT to rats resulted in an increased chemotactic activity of BALF (Gipson et al., 1999). Along similar lines, intravenous truncated (57 amino acid) human elafin (total of approximately 3mg at intervals over a 3 hour period) reduced neutrophil recruitment in a hindlimb ischaemia-reperfusion model in rats (Crinnion et al., 1994). The same elafin preparation (intravenous bolus of 0.67mg, followed by approximately 2.15 mg/h for 2-3 hours) significantly reduced neutrophil recruitment to the heart in a rat model of myocardial infarction or repetitive myocardial ischaemia (Tiefenbacher et al., 1997). In a more recent study, human elafin cDNA (identical to that described in this work) was incorporated into Sendai virus, and the resulting complex used to transfect excised, homologous jugular vein grafts from rabbits *ex vivo*. The transfected segments were grafted into an excised portion of the right common carotid artery (O'Blenes et al., 2000). This manipulation resulted in a reduction in graft neutrophilia (O'Blenes et al., 2000).

In summary, elastase would appear to have at least some role in neutrophil migration. While SLPI may have little effect on this process *in vitro*, α_1 -PI appears to have a pleiotropic role, possibly suggesting an inhibitory effect for the native molecule but a stimulatory effect for digested, complexed and oxidised forms as might be found in the setting of inflammation. However *in vivo* studies to date suggest an inhibitory effect of low molecular weight elastase inhibitors on neutrophil migration.

In returning to the question of whether elafin had a direct effect on LPS-mediated neutrophil migration in this study, certain points relating to the existing literature require attention. The first thing to note is that no other data exist with regard either to the effect of elafin on neutrophil migration to the lung *in vivo*, or to its effect *in vitro*. Secondly, the *in vivo* studies using elafin (Crinnion et al., 1994; Tiefenbacher, 1997; O'Blase et al., 2000) and SLPI (Suzuki et al., 1996; Gipson et al., 1999) were fundamentally different from the experiments in this chapter, in that the effect of elastase inhibitors was assessed in the setting of conditions known to produce significant tissue damage in control animals (secondary to vascular pathology in the case of the elafin studies). In experiments described here little if any tissue damage was induced by 0.5µg of LPS in control mice, as assessed histologically (Figure 6) and by protein concentrations in BALF (Figure 5). In other words the intention of the experiments described here was simply to determine the effect of elafin augmentation on LPS-mediated neutrophil migration into previously healthy lung, whereas the intention of the other studies described was to determine whether elafin (or SLPI) had tissue protective effects. Furthermore, the studies by Crinnion et al. (1994) and Tiefenbacher et al. (1997) used truncated elafin at concentrations far higher than those achieved here.

Some circumstantial evidence in this chapter implies that the effect of elafin on neutrophil migration was not direct. For example, if human elafin exerted a specific chemotactic activity, Ad-elafin/PBS mice might have been expected to have higher numbers of neutrophils in BALF than Ad-lacZ/PBS mice, and a significant correlation might have been expected between the elafin concentration and neutrophil count in Ad-elafin/LPS mice. Neither of these conditions were satisfied. Furthermore if elafin exerted its effect through anti-elastase activity, one might have expected BALF from Ad-elafin/LPS mice to have augmented activity against HNE, which proved not to be the case. Finally recombinant elafin did not exert chemotactic activity *in vitro* (though it must be recalled that the moiety used was a truncated form of elafin).

In conclusion, no evidence has arisen specifically to support a direct action of elafin on neutrophil migration, either with regard to anti-protease activity or a direct chemotactic effect. However an as yet unidentified direct mechanism cannot be excluded at this stage. In this context two specific theoretical possibilities are worthy of brief consideration. The first stems from literature suggesting a pro-inflammatory

role for fragmented or complexed α_1 -PI, and contends that elafin complexes or fragments may exert a similar effect. Although the antibody used in this study can (with reduced efficiency) recognise elafin incubated with HNE (section 3.2.2.), there is no evidence to say whether it recognises elafin complexed to murine elastase. A second intriguing possibility is suggested by experiments showing that elastase and elastase inhibitors may specifically interact with the presentation of LPS to inflammatory cells. In particular SLPI is capable of binding LPS (Ding et al., 1999), and HNE can degrade CD14 (Le-Barillec et al., 1999). It could therefore be argued that the effects reported here are due to inhibition of elastase-mediated proteolysis of CD14 and/or to a chemotactic effect of elafin/LPS complexes. Such mechanisms would reconcile the fact that a pro-migratory action of elafin has not been noted in *in vivo* systems from which LPS is absent, and this area deserves further study. However it is insufficient to explain the small pro-migratory effect of human elafin *in vitro*.

4.3.2.3. Is Ad-elafin's effect on LPS-induced neutrophilia mediated by an indirect action of human elafin?

If human elafin enhances neutrophil migration in the murine lung partly or wholly via an indirect mechanism, then it seems most likely that an effect of elafin on the expression and/or function of neutrophil chemoattractants is involved. A number of well characterised murine neutrophil and macrophage chemokines have been described (reviewed by Haelens et al., 1996). Given the 24-hour exposure to LPS in the experiments described here, and the limited quantity of BALF available from each mouse, it was decided to concentrate primarily on the C-X-C neutrophil chemokine MIP-2, then use remaining BALF to study the other molecules listed in Table 3.

The data indicate that the pattern of MIP-2 generation was broadly in keeping with the pattern of airway neutrophilia after exposure to LPS (ie the concentration of MIP-2 was higher in Ad-elafin mice than in PBS mice than in Ad-lacZ mice (Figure 5)). Interestingly, adenovirus transfection *per se* seemed to be associated with a down-regulation of MIP-2 secretion. These data could be taken to infer a role for elafin in stimulating MIP-2 expression directly. There are however strands of evidence opposing this possibility. Firstly, the correlation between MIP-2 expression and elafin production was not statistically significant. Secondly, subsequent experiments performed in this laboratory by Dr Jean-Michel Sallenave and Ms.

Bianca Hiemskerk showed that MIP-2 was not up-regulated from Ad-elafin transfected murine Clara cells exposed to LPS.

A very similar situation exists when considering TNF- α , which (in pooled samples) also mirrored airway neutrophilia after LPS administration in being present in higher concentrations in BALF from Ad-elafin/LPS mice than from PBS/LPS mice than from Ad-lacZ/LPS mice. Dr Sallenave and Ms. Hiemskerk showed that TNF- α was down-regulated from Ad-elafin transfected murine alveolar macrophages exposed to LPS.

MIP-1 α is known to be secreted preferentially by alveolar macrophages in response to LPS, and is both a macrophage and neutrophil chemokine (VanOtteren et al., 1994; Standiford et al., 1995). The pattern of expression of MIP-1 α in pooled BALF paralleled the BALF macrophage and neutrophil counts in this study (Figure 5 and Table 4). This could potentially be in keeping with a role for Ad-elafin in augmenting LPS-induced monocyte migration also.

What is clear from these data is that Ad-elafin appears to be associated with up-regulation of more than one chemokine after LPS administration. Dr Sallenave and Ms. Hiemskerks' data suggest that elafin itself is unlikely to stimulate the genes for MIP-2 and TNF- α directly, and the absence of neutrophilia in Ad-elafin/PBS mice (Figure 5) would tend to support this view. One can do little more than speculate as to potential mechanisms involved, but it is again tempting to invoke a situation whereby elafin leads to more efficient presentation of the inflammatory stimulus (ie LPS) leading to an appropriate up-regulation of inflammatory chemokines. With this and the previously noted proteolysis of CD14 in mind (Le-Barillec et al., 1999), it is intriguing that TNF- α production was found to be CD14-dependent in a rabbit model of pulmonary LPS exposure (Ishii et al., 1993).

Irrespective of the mechanism of Ad-elafin's augmentation of LPS-induced neutrophilia, it is worth emphasising that the doubling of airway neutrophilia observed was not associated with an increase in protein leak across the alveolar-epithelial barrier (Figure 5). The dissociation of neutrophil influx and epithelial permeability after administration of LPS to the lungs has been described elsewhere in mice (Chignard and Balloy, 2000), rats (Yang et al., 1998) and humans (Sandstrom et al., 1992). The specific implication for this body of work relates to the apparent safety of IT Ad-elafin *in vivo*.

Turning now to the effect of Ad-elafin on neutrophil migration *in vitro*, an indirect effect of elafin may also explain the small pro-migratory effect described in Figure 10, as recombinant human elafin itself had no effect. However it must be recalled that the moiety used was truncated. Interestingly the small pro-migratory effect induced by Ad-elafin was not attributable to augmentation of IL-8 from A549 cells, as higher levels of IL-8 were consistently found in supernatants from untransfected cells (Figure 11). Furthermore, IL-8 seemed to contribute relatively little to neutrophil migration in this system, as anti-IL-8 antibodies made little impression on rates of migration. In this setting, while the *in vitro* experiments were supportive of a role for Ad-elafin in augmenting neutrophil migration, the principal conclusion is that elafin's effect is likely to be via stimulation of endogenous neutrophil chemoattractants, but the nature of these remains undefined.

In contrast to experiments performed in mice, a clear effect of adenovirus itself was present *in vitro* (Figure 10). It seems likely that the chemoattractant(s) induced by adenovirus differs from that induced by elafin, as anti-elafin antibody resulted in equivalent levels of chemoattractant activity from Ad-lacZ and Ad-elafin transfected cells which remained far in excess of those from untransfected cells. Amin et al. (1995) also showed that adenoviral transfection of A549 cells results in enhanced neutrophil chemoattractant activity, but in contrast to the observations in Figure 11 they found that transfected cells produced higher concentrations of IL-8 than did untransfected cells. Furthermore in their hands IL-8 was responsible for virtually all of the chemoattractant activity (Amin et al., 1995). The results described here in Figure 11 have been reproduced in the absence of neutrophils several times in this lab (ie Ad-lacZ transfected cells consistently produce less IL-8 than do untransfected cells), and these results are therefore markedly at odds with those of Amin et al. (1995). The reason for this discrepancy remains unclear. A separate group found no significant change in IL-8 secretion from Ad-lacZ transfected human bronchial epithelial cells (Noah et al., 1996).

To summarise this section, low dose Ad-elafin delivered by the IT route consistently produces human elafin in murine airways without inducing overt injury. Both epithelial cells and inflammatory cells appear to be transfected. Human elafin expression is significantly up-regulated in the presence of LPS and in turn Ad-elafin transfection is associated with a significant augmentation in LPS-induced neutrophilia. The precise mechanism by which elafin effects this augmentation

remains unclear. The pro-migratory effect of Ad-elafin on neutrophils can be extended to human epithelial cells *in vitro*.

4.3.3. The effect of LPS on elafin secretion from human epithelial cells and macrophages.

As described previously, the demonstration of LPS-inducible transgene expression in mice led to studies aimed at determining whether this effect was reproducible in human cells. A549 cells and peripheral blood monocyte-derived macrophages were studied in view of their ready accessibility, and because the cells likely to encounter IT adenovirus first are considered to be epithelial cells and macrophages.

4.3.3.1. The effect of LPS on adenovirus-transfected A549 cells.

The significant up-regulation of elafin from untransfected A549 cells exposed to LPS (Figures 12 and 13) indicates that elafin secretion is responsive both to exogenous inflammatory mediators and to endogenous early cytokines such as IL-1 and TNF (Sallenave et al., 1994). Conversely, elafin secretion from A549 cells is reduced by the application of HNE (Reid et al., 1999). The mechanisms by which elafin secretion is up-regulated by LPS remain to be elucidated. While a stimulatory effect on the endogenous elafin promoter may be inferred from data in this chapter, Bingle et al. (2001) described no effect of LPS on elafin mRNA expression from primary type II alveolar epithelial cells.

Importantly from the point of view of these specific experiments however, it can be confidently stated that adenoviral transfection *per se* does not augment elafin secretion in response to LPS. A further technical point is that basal elafin secretion does not appear to be markedly influenced by the presence or absence of serum, constitutive levels being similar in Figures 12 and 13 in this chapter and in Figure 3, Chapter 3.

The pattern of elafin secretion from Ad-elafin treated cells exposed to LPS was quite different to that from control cells (Figures 12 and 13). It seems likely that the relative unresponsiveness to LPS of cells transfected with moi 50 reflects a near maximal stimulation of elafin secretion by A549 cells even in the absence of LPS (Figure 12). In contrast, in cells treated with moi 1, the LPS-dependent increase in

elafin secretion over and above that seen in control cells can be assumed to result from stimulation of the mCMV promoter. As such the mCMV-elafin transgene complex is sensitive to LPS both in human cells *in vitro* and in murine lungs. However it should be noted that relatively high concentrations of LPS (1 µg/ml) were required to stimulate a small up-regulation *in vitro*, in contrast to the striking response seen *in vivo* (Figure 7). This difference is likely to reflect an action of numerous LPS-induced inflammatory mediators on the mCMV promoter in the more complex *in vivo* situation.

4.3.3.2. The effect of LPS on adenovirus-transfected monocyte-derived macrophages.

The results of this study suggest that human monocyte-derived macrophages are capable of producing elafin, but this was only induced after stimulation with high doses of Ad-lacZ and LPS. A separate group has recently described the presence of human elafin mRNA in stimulated alveolar macrophages but not in peripheral blood monocytes (Mihaila and Tremblay, 2001).

A potential criticism of the experiments in this chapter is that peripheral blood monocyte-derived macrophages were used instead of alveolar macrophages. There is good evidence to suggest that macrophage populations derived from different biological compartments have different phenotypic characteristics (Campbell et al., 1991; VanOtteren et al., 1994), possibly relating, in the case of alveolar macrophages, to different prevailing oxygen tensions (Peters-Golden et al., 1990). For the purposes of this study, blood-derived macrophages were felt to be of sufficient interest for a number of reasons. Firstly, as discussed later in this section, adenoviral transfection efficiency of alveolar and peripheral blood monocyte-derived macrophages is thought to be similar. Secondly, in the broader context of gene therapy, *ex vivo* transfection of either population is a legitimate target. Thirdly, from a purely practical viewpoint, peripheral blood monocyte-derived macrophages are considerably more accessible, using a much less invasive technique.

In contrast to the situation described in control cells, Ad-elafin was capable of generating elafin in macrophages from at least some donors, and the mCMV promoter appeared to be sensitive to LPS in these macrophages (Figure 15). The factors determining which donors' macrophages express elafin remain unclear.

The rate of macrophage transfection by adenovirus in this study closely approximates to that described elsewhere for both monocyte-derived macrophages (Haddada et al., 1993; Schneider et al., 1997) and alveolar macrophages (Worgall et al., 1999a). Indeed the marked discrepancy in transfection efficiency of macrophages (Figure 14) and A549 cells (Figure 1, Chapter 3) has been described elsewhere and attributed to the differences in expression of the CAR receptor on the two cell types (Kaner et al., 1999).

Similarly, the factors determining macrophage transfection efficiency *in vivo* require to be further characterised. Macrophages effect rapid clearance of adenovirus administered IT, and in so doing initiate an immune response (Kuzmin et al., 1997; Worgall et al., 1997b; Zsengellér et al., 2000). However there is little doubt that some macrophages allow expression of transgene *in vivo* for several days. Immunochemical staining in this study identified occasional macrophages expressing human elafin 6 days after IT transfection (Figure 9). This is in keeping with, for example, expression of human transgene in rat alveolar macrophages 3 days after IT instillation (Danel et al., 1998) and of labelled murine transgene in alveolar macrophages at least 2 weeks after *ex vivo* transfection (Worgall et al., 1999a).

The importance of these issues relates to whether macrophages represent a useful target for gene therapy in the lung, whether they be transfected *in vivo* or *ex vivo*. The data in Figure 16 could suggest that under the appropriate conditions, transgene expression can be markedly up-regulated *ex vivo*. These data find some resonance in two recent elegant studies. One of these described functional pulmonary expression of transgene after sequential *ex vivo* transfection of alveolar macrophages and IT reconstitution (Worgall et al., 1999a). The other described *ex vivo* transfection of monocyte-derived macrophages resulting in functional correction of the characteristic genetic defect of X-linked chronic granulomatous disease (Schneider et al., 1997).

If the macrophage is to be a useful target for gene therapy, then transfection efficiency will need to be improved. The scope certainly exists to achieve this in that simply applying adenovirus to cells in suspension (as opposed to adherent cells) increases efficiency approximately 3-fold (Schneider et al., 1997), while work performed by Dr P. Henriksen in this lab has demonstrated that incubation with calcium phosphate precipitate increases efficiency still further. In tandem with these

observations, novel receptor-targeting offers alternative routes of transgene delivery to macrophages (Ferkol et al., 1998).

To summarise this section, and place it in the context of previous sections, the constitutive expression of elafin in pulmonary epithelial cells is up-regulated by LPS, while neither constitutive nor LPS-inducible elafin could be detected in untransfected peripheral blood monocyte-derived macrophages. Ad-elafin transfection of both A549 cells and macrophages resulted in increased elafin secretion in response to LPS, apparently attributable to a stimulatory effect on the powerful mCMV promoter.

4.4. SUMMARY

The data in this chapter have demonstrated efficient, consistent, and LPS-inducible expression of human elafin in the murine lung using Ad-elafin at doses which preclude significant vector-induced neutrophilia or epithelial disruption. These conditions suggested a potential role for Ad-elafin in preventing tissue injury in animal models.

Elafin is a low molecular weight, cationic inhibitor of HNE (and proteinase 3) expressed principally at epithelial surfaces subject to environmental exposure such as the airways, skin and oral cavity (Wiedow et al., 1990; Sallenave and Ryle, 1991; Nara et al., 1994). Secretion may be rapidly up-regulated by early inflammatory cytokines (Sallenave et al., 1994) or by LPS. Elafin appears to be able to augment LPS-induced neutrophil recruitment without increasing epithelial permeability, and furthermore can protect pulmonary epithelium against the deleterious effects of HNE liberated extracellularly. From a teleological viewpoint, this combination of properties strongly suggested a physiological role for elafin in early innate immunity. Given that the immune system has evolved to protect against microbial pathogens, and that several endogenous antibiotics are small cationic peptides (reviewed by Hancock, 1997), the hypothesis was formed that elafin may have direct antimicrobial activity against pulmonary pathogens.

CHAPTER 5

ANTIMICROBIAL EFFECTS OF ELAFIN

5.1. AIMS AND BACKGROUND

The primary aim of the work described in this chapter was to determine whether elafin has intrinsic antimicrobial activity. In the event of an antimicrobial effect being demonstrated then secondary aims were to determine whether protein folding was a prerequisite for antimicrobial function, and to determine the relative contributions of the distinct structural domains of elafin.

The various forms of elafin used in these experiments have been described in Chapter 2 (sections 2.1.1.2. and 2.1.1.3.). In brief, they included recombinant human elafin (approximately 6 kDa) comprising the COOH-terminal end of the molecule, and a range of synthetically engineered molecules. The synthetic molecules included full length folded elafin (approximately 9.9 kDa), full length unfolded elafin, the NH₂-terminal domain (approximately 5.2 kDa) and the COOH-terminal domain (approximately 4.8 kDa). For comparative purposes, recombinant human SLPI (approximately 11.7 kDa) was studied.

Most previous studies assessing the potential antimicrobial effects of peptides have compared bacterial colony counts in the presence of test peptide with those in buffer/vehicle. In the experiments described in this chapter a second control was used in the form of equimolar concentrations of human serum albumin (HSA), to control for any potential non-specific effect of protein. HSA is a circulating anionic molecule which is found in BALF in the context of lung epithelial leak.

The effect of elafin was assessed against two bacteria, namely *P. aeruginosa* and *S. aureus*. These bacteria were selected for four principal reasons. Firstly, both are important pulmonary pathogens, particularly in the context of CF (reviewed by Govan and Nelson, 1992; Hutchison and Govan, 1999) and in the setting of immune compromise (Maurer et al., 1992; Wade et al., 1995). Secondly, it seemed desirable to test elafin against both Gram positive (*S. aureus*) and Gram negative (*P. aeruginosa*) organisms. Thirdly, as one of the major challenges facing medicine is the development of novel strategies to combat antimicrobial resistance, it seemed pertinent to study two bacteria with a propensity for clinically important resistance

mechanisms (reviewed by Hancock, 1998; Waldvogel, 1999). Finally, a wealth of experience in the biology of these organisms exists locally within related research groups interested in CF (Davidson et al., 1995; Govan and Deretic, 1996). The following sections briefly discuss *P. aeruginosa* and *S. aureus*, with a little more emphasis on the former as *P. aeruginosa* features prominently in Chapter 6.

5.1.1. *Pseudomonas aeruginosa* strain PAO1.

P. aeruginosa is a ubiquitous environmental nonfermentative Gram negative bacillus. *P. aeruginosa* may cause pulmonary infection in the setting of breached host defence, for example in ventilator-associated pneumonia (Meduri et al., 1998) or after lung transplantation (Maurer et al., 1992). However, with regard to pulmonary disease, *P. aeruginosa* is most closely associated with CF, resulting in colonisation of over 90% of patients by adulthood (Davis et al., 1996; Hutchison and Govan, 1999; Greening, 2000).

Factors governing adherence and internalisation of *P. aeruginosa* to airway epithelial cells have received considerable attention, particularly with regard to differences between CF cells and non-CF cells. Thus it has been suggested that CFTR itself regulates clearance of *P. aeruginosa* in the healthy lung (Pier et al., 1996; Davies et al., 1997), whilst in patients with CF, excessive and inducible expression of asialoGM₁ provides a substrate for adhesion of *P. aeruginosa* (Saiman and Prince, 1993). It is certainly the case that *P. aeruginosa* elaborates a number of potent virulence factors including LPS, exotoxins, haemolysins, and proteases (Vasil, 1986). In addition *P. aeruginosa* secretes a well characterised elastase (Moriyama et al., 1965), though it should be noted that this molecule is a metalloprotease not a serine protease, and as such its structure does not predict for inhibition by elafin, SLPI or α_1 -PI. In addition to the range of virulence factors produced by *P. aeruginosa*, in the lungs of patients with CF this organism has the remarkable capacity to change from a non-mucoid phenotype to a mucoid phenotype characterised by production of alginate and the generation of a complex biofilm which greatly enhances evasion of host defences (Hutchison and Govan, 1999). Colonisation with mucoid *P. aeruginosa* is associated with a poor prognosis (Pedersen et al., 1992), forming the rationale for aggressive strategies aimed at eradication of the organism at an early (non-mucoid) stage in the disease (Valerius et al., 1991; Frederiksen et al., 1997).

In conjunction with the inherent virulence of the organism, eradication of *P. aeruginosa* is also hindered in clinical practice by high levels of intrinsic resistance to commonly used antimicrobial agents (Hancock, 1998). Resistance is mediated principally through reduced outer membrane permeability of the organism, though antibiotic efflux pumps and induction of enzymes capable of degrading antimicrobials also contribute. The combination of antimicrobial resistance and unique colonisation characteristics renders effective clearance of *P. aeruginosa* extremely difficult.

The strain of *P. aeruginosa* used in this work (PAO1) is the best characterised of all laboratory strains, and indeed the complete genome sequence has recently been characterised (Stover et al., 2000). PAO1 was originally isolated from a skin wound in a patient in Melbourne, Australia, and is non-mucoid in phenotype (Holloway, 1955). Initial colonisation of the lung in CF is with non-mucoid strains (Govan and Deretic, 1996). PAO1 is known to generate pulmonary pathology in rodent models (George et al., 1993), and stimulates elastase release in hamster airways (Melby et al., 1985).

5.1.2. *Staphylococcus aureus* strain C1705.

S. aureus is a Gram positive coccus which has long been recognised to have an association with abscess formation and sepsis (reviewed by Lowy, 1998). *S. aureus* can cause a severe, cavitating form of community acquired pneumonia, often in patients with pre-existing debility. It is also closely associated with lung disease in CF (Govan and Nelson, 1992).

In keeping with the themes developed for *P. aeruginosa*, *S. aureus* produces potent virulence factors, for example toxins, coagulase, proteases and hyaluronidase. In addition, *S. aureus* is increasingly characterised by the emergence of antimicrobial resistance. Thus while β -lactamase expression may eradicate susceptibility to penicillin, the presence of the *mec* gene confers methicillin-resistance and consequently greatly reduced therapeutic options (Chambers, 1997). This alarming clinical situation has been compounded by the emergence of isolates resistant to vancomycin (Hiramatsu et al., 1997). The strain used in this study is a clinical isolate from a patient with CF.

5.2. RESULTS

5.2.1. Preliminary experiments.

Both PAO1 and C1705 grew efficiently in TSB with logarithmic phase growth for each strain occurring at around 3 hours (Figure 1). This time point was therefore used to establish growth in subsequent experiments.

The remaining requirement was to establish whether synthetically generated full-length folded elafin was functionally active. This was assessed by measuring the anti-elastase activity of the molecule and its derivatives (Figure 2). Full-length folded elafin, the synthetic COOH-terminal domain of elafin and recombinant elafin all inhibited HNE, the elastase inhibitory activity of each moiety being strikingly similar (Figure 2). A slightly higher elastase inhibitory activity was observed for recombinant SLPI (Figure 2). In contrast the NH₂-terminal domain of elafin and full-length unfolded elafin had no detectable anti-elastase activity (Figure 2).

Finally, neat aliquots of each of the synthetic moieties, recombinant elafin, recombinant SLPI, and HSA were plated out on Columbia agar and incubated overnight at 37°C. No growth was detected for any of the species used, effectively excluding bacterial contamination of the test substances.

5.2.2. The effect of elafin on *P. aeruginosa* PAO1.

Elafin treatment resulted in significantly fewer PAO1 colonies than did either of the controls used (Figure 3). The effect of elafin was significant throughout the dose range studied (1-25µM) and maximal at 2.5µM. At this concentration, eradication of PAO1 was almost complete. There was an inverse dose-response relationship over the range 2.5-25µM. The effect of elafin appeared to be bactericidal, at least in part, as for all concentrations of elafin (1-25µM) the colony count from samples taken at the end of 2 hours incubation with PAO1 was significantly lower than the count from samples containing PAO1 and phosphate buffer plated out prior to the 2 hour incubation ($p < 0.01$ for all concentrations of elafin).

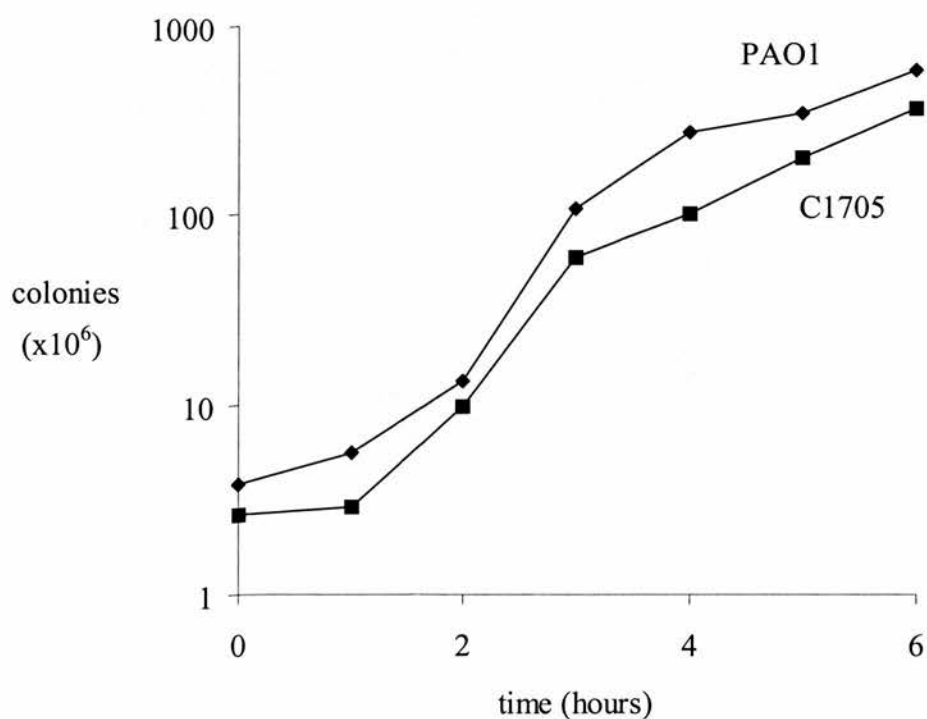


Figure 1. Growth curves for PAO1 and C1705.

Samples of PAO1 and C1705 were incubated in TSB overnight. Aliquots of the resulting suspensions were diluted 100 times in fresh TSB and incubated at 37°C. Aliquots were removed at hourly intervals, plated out on agar, and incubated overnight at 37°C prior to estimation of colony counts.

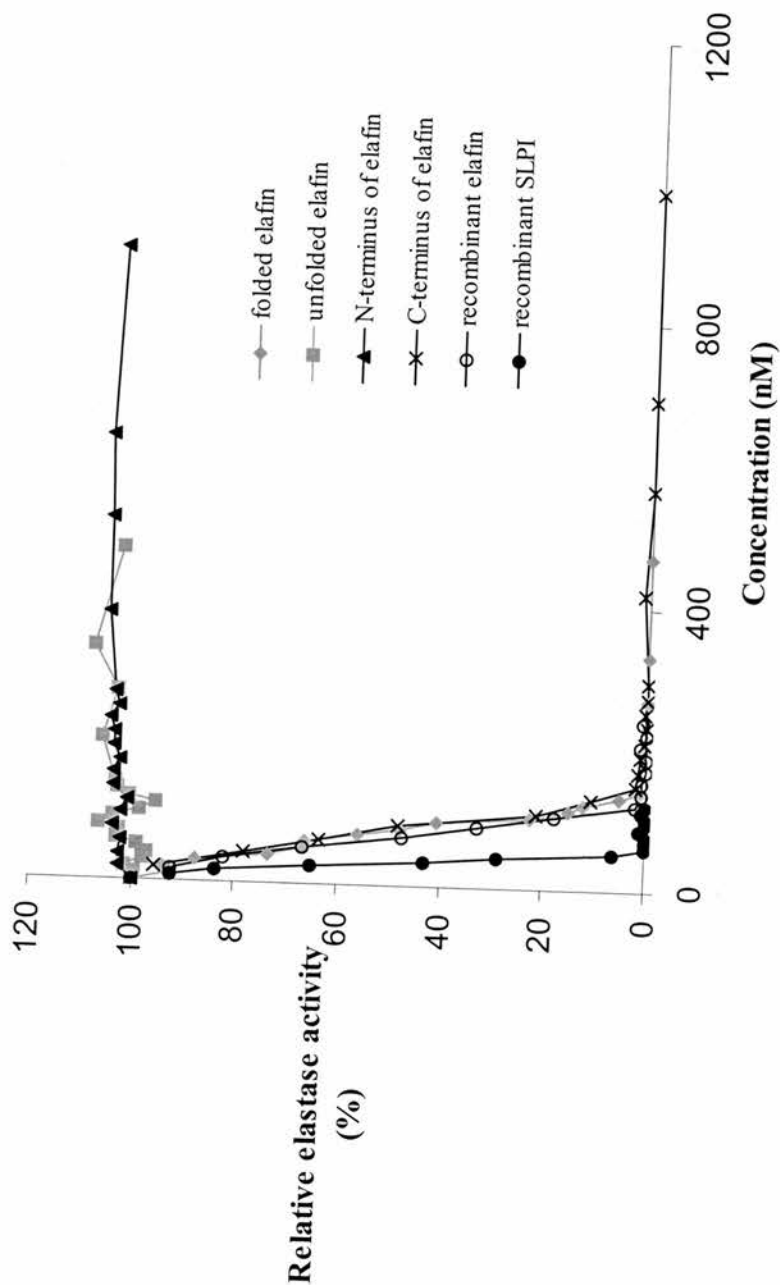


Figure 2. Elastase inhibitory activity of synthetic elafin fragments, recombinant human elafin and recombinant human SLPI. Increasing concentrations of folded full length elafin, unfolded full-length elafin, NH_2 -terminal domain of elafin, COOH -terminal domain of elafin, recombinant human elafin or recombinant human SLPI were added to HNE (67 nM), and incubated at 37°C for 15 minutes. HNE-specific chromogenic substrate was added and absorbance read at 405nm as a function of time.

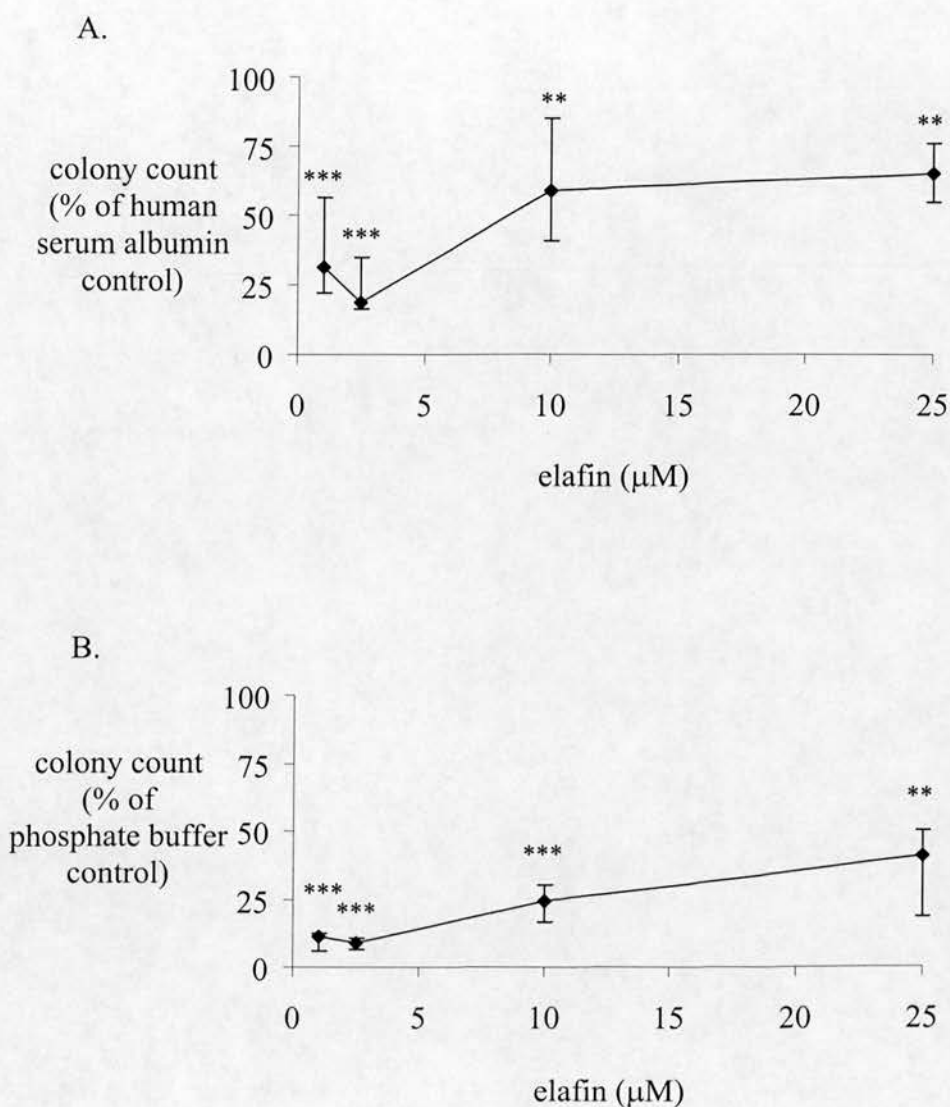


Figure 3. Elafin has antimicrobial activity against *Pseudomonas aeruginosa* PAO1.

100μl of a suspension of PAO1 was incubated in TSB at 37°C for 3 hours, washed and resuspended in phosphate buffer. 30μl aliquots of PAO1 suspension were then incubated with 90μl of elafin in concentrations ranging from 1-25μM in phosphate buffer, using either 90μl of HSA (1-25μM in phosphate buffer; A) or phosphate buffer alone (B) as control. Samples were incubated for 2 hours at 37°C then plated on Columbia agar and incubated overnight at 37°C prior to colony counting.

In the phosphate buffer control the median concentration of PAO1 was approximately 79,600 cfu/ml.

Data points represent medians and interquartile ranges, n = 8-13 for each data point.

** = significant difference, $p < 0.01$, compared with control. *** = significant difference, $p < 0.001$, compared with control.

Figures 4 and 5 illustrate the effects of different elafin moieties against *P. aeruginosa*. Several points arise. Importantly the HSA control also appeared to have some antimicrobial activity relative to phosphate buffer (Figure 4). However this effect was considerably less than the effect of equimolar concentrations of full-length folded elafin (Figures 4 and 5).

At low concentrations (1 and 2.5 μ M) the full-length folded elafin molecule had considerably greater antimicrobial activity than either the COOH- or NH₂-terminal domains. However the NH₂-terminal domain had at least as powerful antimicrobial activity as the full-length folded molecule at higher concentrations (Figures 4 and 5). Interestingly, while unfolded elafin and the NH₂-terminal domain had no demonstrable anti-elastase activity, they each had some antimicrobial activity (Figures 2 and 4). A further important point is that the dose-response curves for all moieties tested (with the exception of the NH₂-terminal domain) appeared to be parallel.

When compared directly with recombinant human SLPI in 3 separate experiments, elafin appeared to have an equivalent effect at 1 μ M, and a greater effect at 2.5 and 10 μ M (Figure 6). However these results should be interpreted with some caution as the average activity of elafin against PAO1 in the experiments described in Figure 6 was slightly higher than the average activity in the experiments described in Figures 3B and 4. This apparent discrepancy is explained by the variability inherent to these experiments (as reflected in the error bars in Figure 3). It seems reasonable to conclude that elafin and SLPI had approximately equivalent activity against PAO1 across the concentration range tested.

In a single separate experiment, 0.5 μ M full length folded elafin and 0.5 μ M SLPI were added together to PAO1, and the effect compared with that of either 1 μ M elafin or 1 μ M SLPI respectively. The purpose of this experiment was to determine whether there was synergy in the bactericidal effect of the molecules but this was not demonstrated (the colony count relative to that in phosphate buffer was 11% in the presence of elafin alone, 17% in the presence of SLPI alone, and 13% in the presence of both molecules).

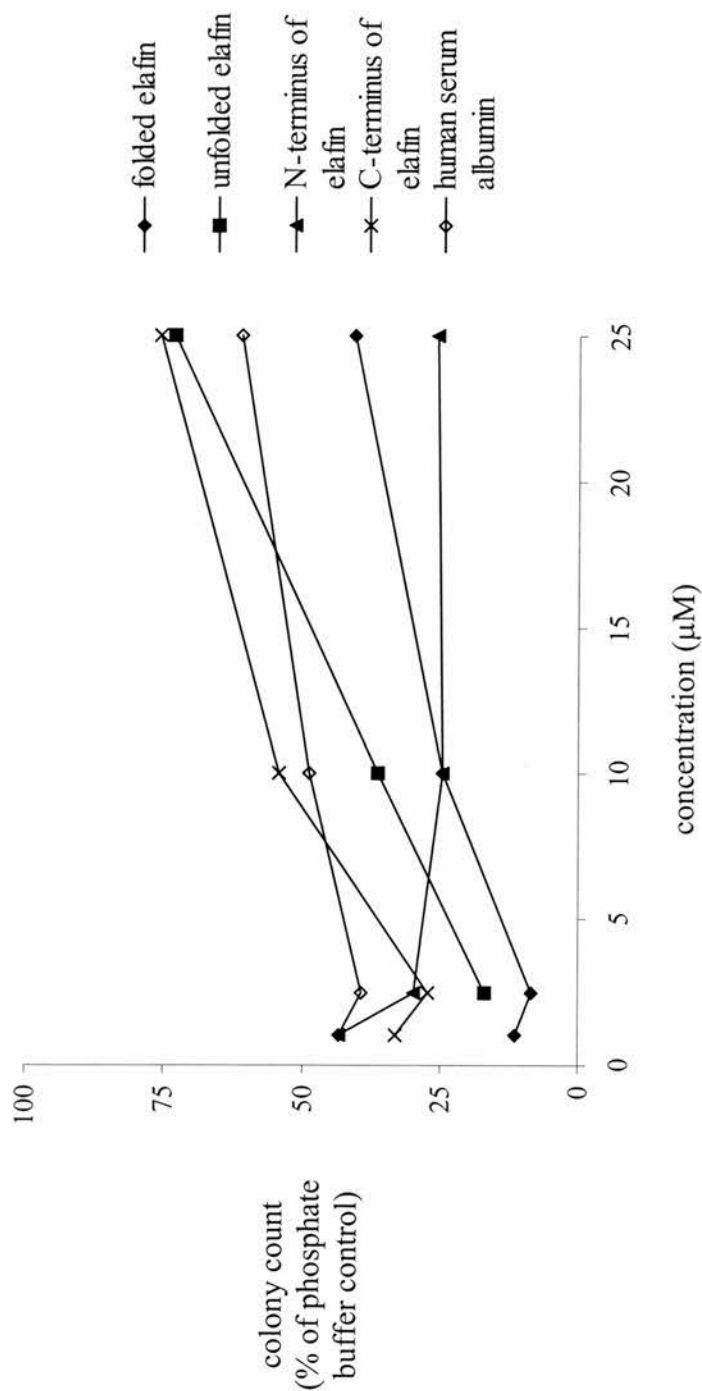


Figure 4. The antimicrobial activity of elafin and elafin fragments against *Pseudomonas aeruginosa* PAO1, relative to control (phosphate buffer).

100μl of a suspension of PAO1 was incubated in TSB at 37°C for 3 hours, washed and resuspended in phosphate buffer. 30μl aliquots of PAO1 suspension were then incubated with 90μl of folded full-length elafin, unfolded full-length elafin, NH₂-terminal domain of elafin, COOH-terminal domain of elafin or HSA (each 1-25μM in phosphate buffer). 90μl of phosphate buffer acted as control. Samples were incubated for 2 hours at 37°C then plated on agar and incubated overnight at 37°C prior to colony counting. Results are expressed relative to the colony count in phosphate buffer (in which the median concentration of PAO1 was approximately 79,600 cfu/ml). Data represent medians from 8-13 separate experiments.

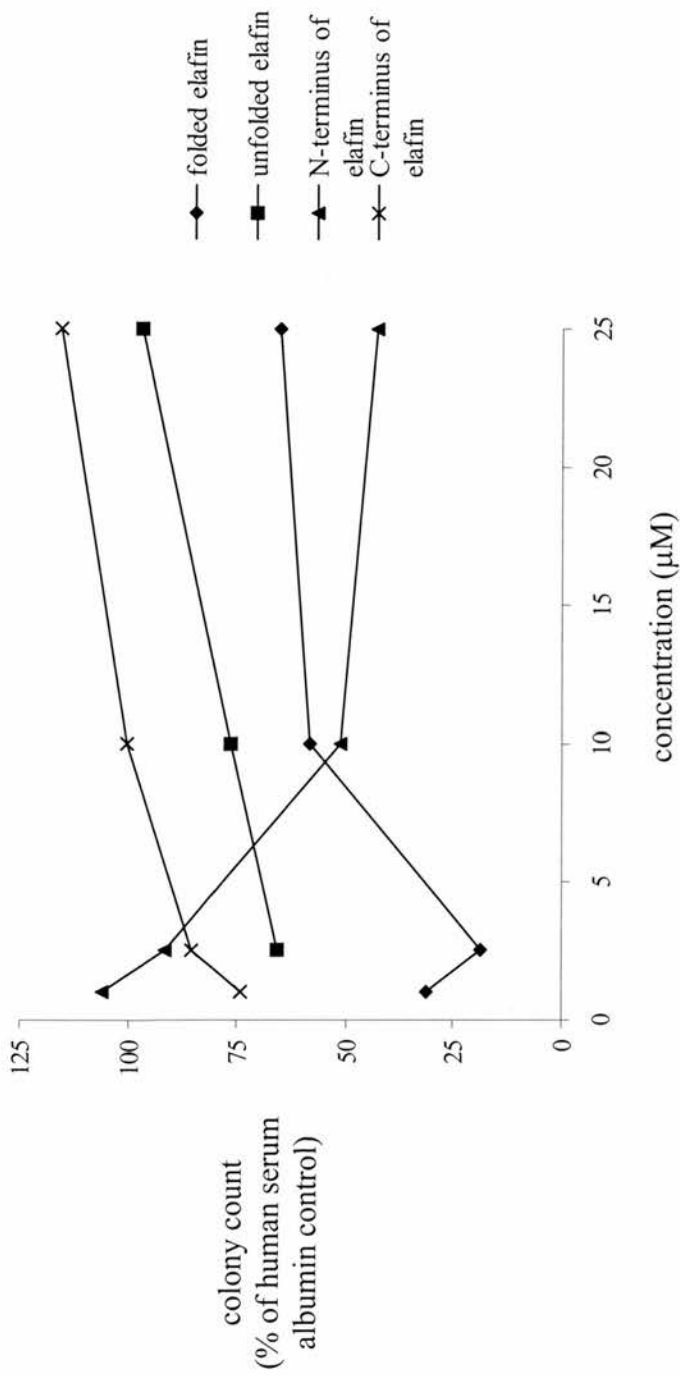


Figure 5. The antimicrobial activity of elafin and elafin fragments against *Pseudomonas aeruginosa* PAO1, relative to control (human serum albumin). 100μl of a suspension of PAO1 was incubated in TSB at 37°C for 3 hours, washed and resuspended in phosphate buffer. 30μl aliquots of PAO1 suspension were then incubated with 90μl of folded full-length elafin, unfolded full-length elafin, NH₂-terminal domain of elafin, COOH-terminal domain of elafin or HSA (each 1-25μM in phosphate buffer). Samples were incubated for 2 hours at 37°C then plated on Columbia agar and incubated overnight at 37°C prior to colony counting. For each test moiety results are expressed relative to the colony count associated with the equimolar concentration of HSA. Data represent medians from 8-13 separate experiments.

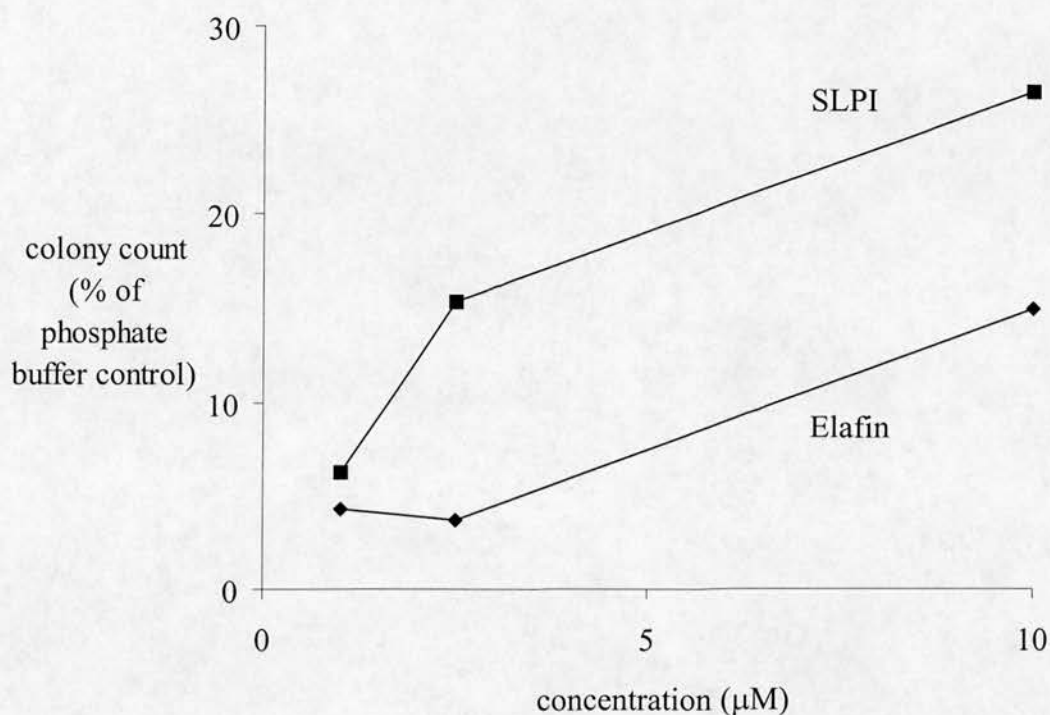


Figure 6. Comparison of the antimicrobial activity of elafin and SLPI against *Pseudomonas aeruginosa* PAO1.

100μl of a suspension of PAO1 was incubated in TSB at 37°C for 3 hours, washed and resuspended in phosphate buffer. 30μl aliquots of PAO1 suspension were then incubated with either 90μl of full-length folded elafin or 90μl of recombinant human SLPI (in concentrations ranging from 1-10μM in phosphate buffer) or 90μl of phosphate buffer alone. Samples were incubated for 2 hours at 37°C then plated on Columbia agar and incubated overnight at 37°C prior to colony counting. Results are expressed relative to the colony count in buffer alone (in which the median concentration of PAO1 was approximately 168,200 cfu/ml).

Data points represent medians, n = 3 for each data point.

5.2.3. The effect of elafin on *S.aureus* C1705.

Elafin also exerted a powerful antimicrobial effect on C1705 (Figure 7). In contrast to the situation described for PAO1, the effect of elafin was generally dose-dependent with no significant effect at 1 μ M, and maximal effect at 25 μ M (Figure 7). Once again the effect appeared to be bactericidal as the C1705 count after 2 hours of incubation with elafin was lower than the count from samples containing buffer and bacteria taken prior to the 2 hour incubation period; this effect reached statistical significance when elafin was applied at 10 and 25 μ M ($p < 0.05$ in each case).

In the case of C1705 full-length folded elafin had greater antimicrobial activity than either the COOH- or NH₂-terminal domains at all concentrations tested over the dose range 2.5-25 μ M (Figures 8 and 9). Indeed while the full-length folded elafin molecule was significantly active against C1705 the NH₂-terminus had no significant effect until a dose of 25 μ M was reached, when the effect remained minimal (Figures 8 and 9). Similarly the COOH-terminus had relatively little effect on C1705 at any of the concentrations tested (Figures 8 and 9).

In contrast unfolded elafin had antimicrobial activity (albeit less than the folded molecule) which closely paralleled that of the folded molecule, at least over the concentration range 1 to 10 μ M (Figures 8 and 9).

In contrast to the situation described for PAO1, HSA appeared to have no antimicrobial effect on C1705. Indeed the counts retrieved from HSA controls were considerably higher than those from controls using phosphate buffer (Figure 8).

When compared directly in 3 separate experiments, neither 1 μ M elafin nor 1 μ M SLPI appeared to have any effect against C1705 (Figure 10). At 2.5 μ M elafin appeared to have no effect, while SLPI had a modest antimicrobial effect. At 10 μ M, both molecules had a modest effect. However, as with the direct comparison of elafin and SLPI using PAO1 these results should be interpreted cautiously, in that on this occasion the effect of elafin described in Figure 10 is slightly less than that described in Figures 7B and 8. On balance the conclusion arising is that elafin and SLPI both have minimal effects on C1705 across the concentration range 1-10 μ M.

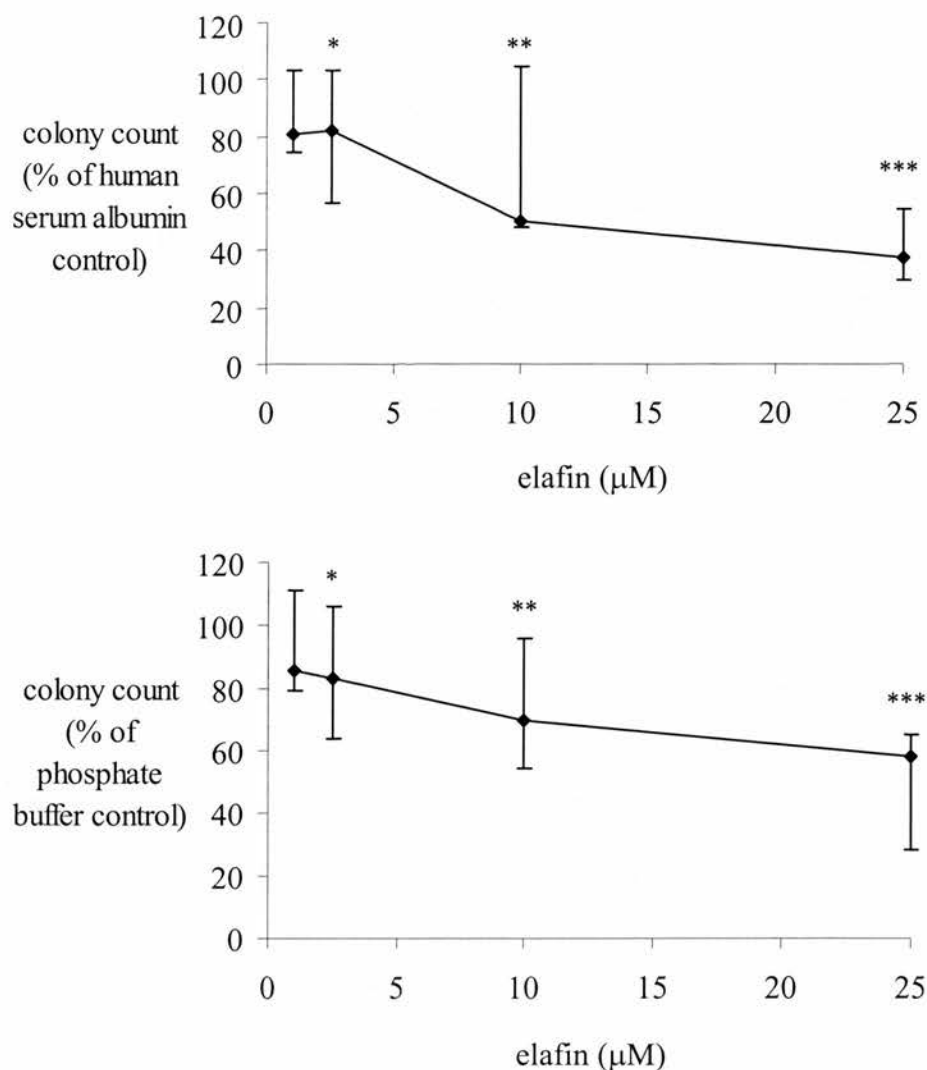


Figure 7. Elafin has antimicrobial activity against *Staphylococcus aureus* C1705.

100μl of a suspension of C1705 was incubated in TSB at 37°C for 3 hours, washed and resuspended in phosphate buffer. 30μl aliquots of C1705 suspension were then incubated with 90μl of elafin in concentrations ranging from 1-25μM in phosphate buffer, using either 90μl of HSA (1-25μM in phosphate buffer; A) or phosphate buffer alone (B) as control. Samples were incubated for 2 hours at 37°C then plated on Columbia agar and incubated overnight at 37°C prior to colony counting.

In the phosphate buffer control the median concentration of C1705 was approximately 25,300 cfu/ml.

Data points represent medians and interquartile ranges, n = 5 for each data point.

* = significant difference, $p < 0.05$, compared with control. ** = significant difference, $p < 0.01$, compared with control. *** = significant difference, $p < 0.001$, compared with control.

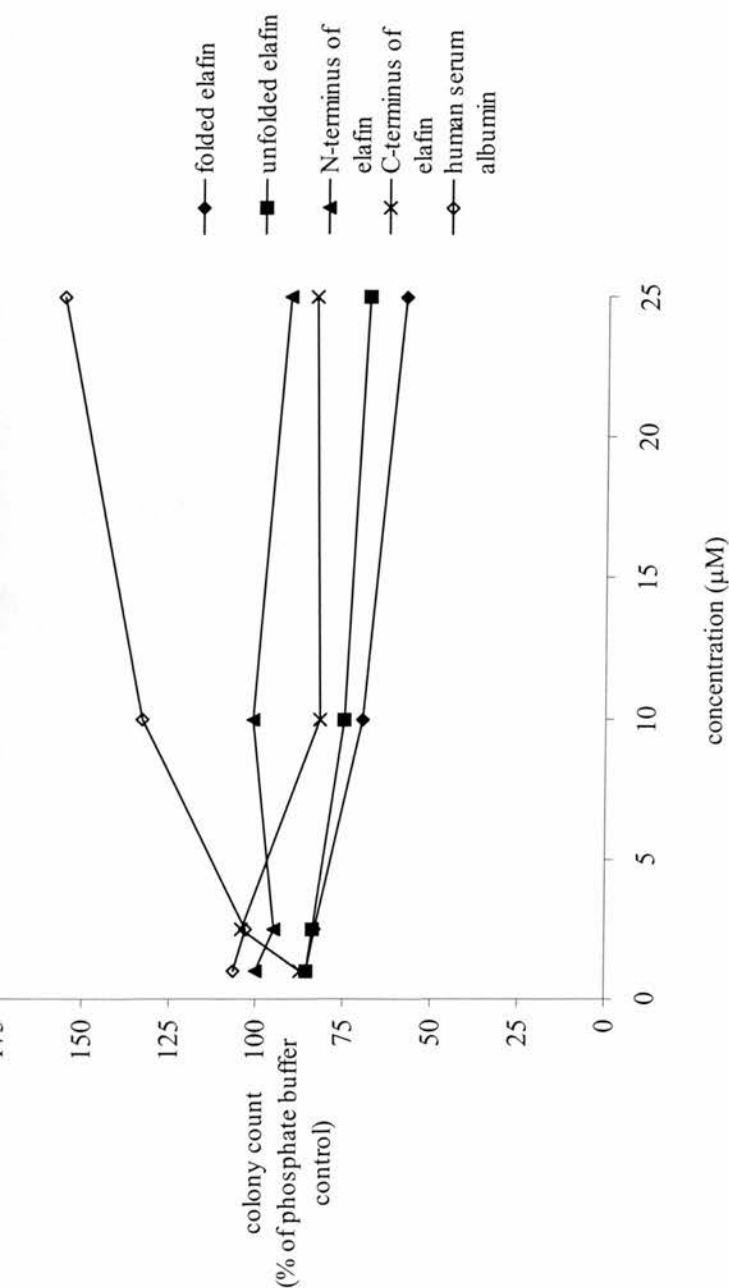


Figure 8. The antimicrobial activity of elafin and elafin fragments against *Staphylococcus aureus* C1705, relative to control (phosphate buffer).

100μl of a suspension of C1705 was incubated in TSB at 37°C for 3 hours, washed and resuspended in phosphate buffer. 30μl aliquots of C1705 suspension were then incubated with 90μl of folded full-length elafin, unfolded full-length elafin, NH₂-terminal domain of elafin, COOH-terminal domain of elafin or HSA (each 1-25μM in phosphate buffer). 90μl of phosphate buffer acted as control. Samples were incubated for 2 hours at 37°C then plated on Columbia agar and incubated overnight at 37°C prior to colony counting. Results are presented relative to the colony count in phosphate buffer (in which the median concentration of C1705 was approximately 25,300 cfu/ml). Data represent medians from 5 separate experiments.

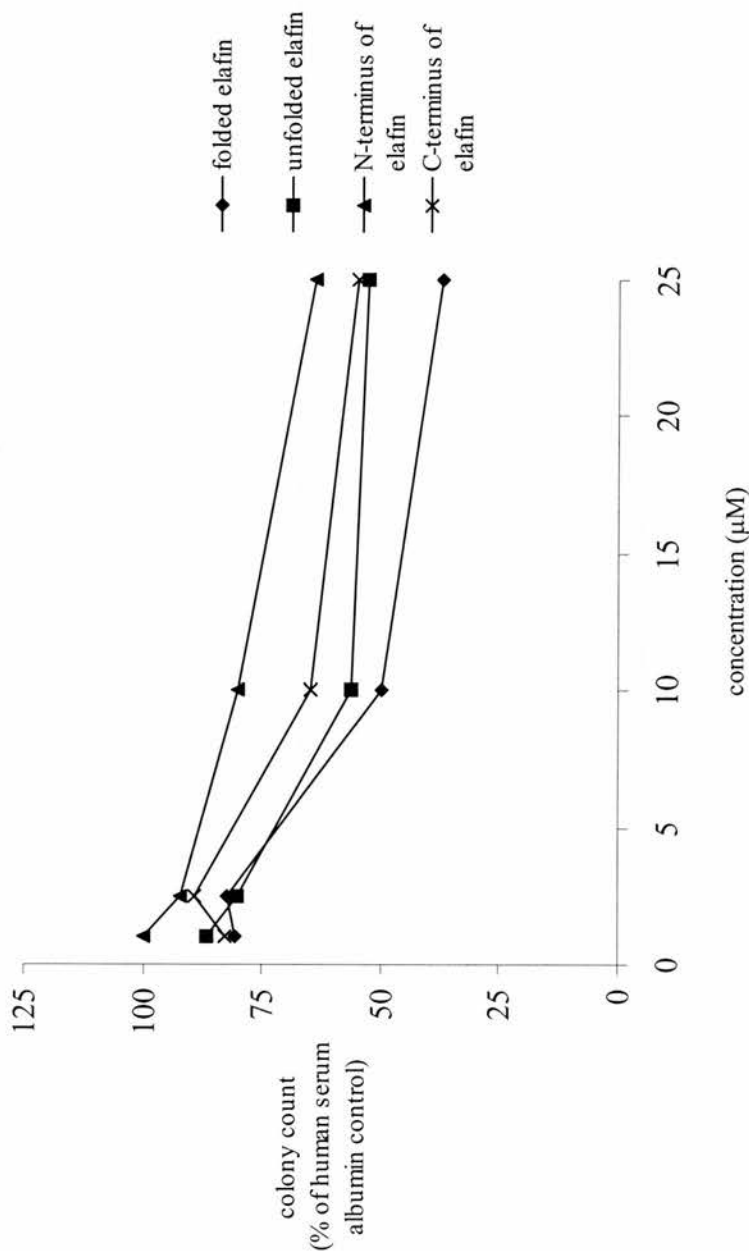


Figure 9. The antimicrobial activity of elafin and elafin fragments against *Staphylococcus aureus* C1705, relative to control (human serum albumin). 100μl of a suspension of C1705 was incubated in TSB at 37°C for 3 hours, washed and resuspended in phosphate buffer. 30μl aliquots of C1705 suspension were then incubated with 90μl of folded full-length elafin, unfolded full-length elafin, NH₂-terminal domain of elafin, COOH-terminal domain of elafin or HSA (each 1-25μM in phosphate buffer). Samples were incubated for 2 hours at 37°C then plated on Columbia agar and incubated overnight at 37°C prior to colony counting. Results are presented relative to the colony count associated with equimolar concentrations of HSA. Data represent medians from 5 separate experiments.

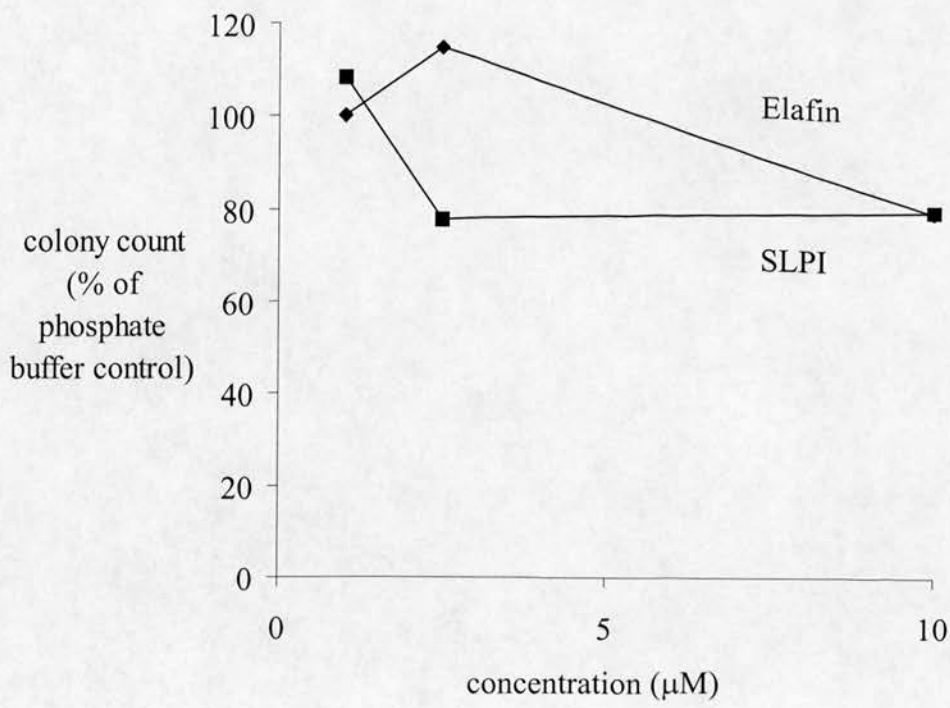


Figure 10. Comparison of the antimicrobial activity of elafin and SLPI against *Staphylococcus aureus* C1705.

100μl of a suspension of C1705 was incubated in TSB at 37°C for 3 hours, washed and resuspended in phosphate buffer. 30μl aliquots of C1705 suspension were then incubated with either 90μl of full length folded elafin or 90μl of recombinant human SLPI (in concentrations ranging from 1-10μM in phosphate buffer) or 90μl of phosphate buffer alone. Samples were incubated for 2 hours at 37°C then plated on Columbia agar and incubated overnight at 37°C prior to colony counting. Results are expressed relative to colony counts in buffer alone (in which the median concentration of C1705 was approximately 30,300 cfu/ml).

Data points represent medians, n = 3 for each data point.

5.3. DISCUSSION

The principal novel observation arising from these data is that elafin has an unequivocal antimicrobial effect against both *P. aeruginosa* and *S. aureus* (Figures 3 and 7).

The availability of elafin fragments and unfolded elafin allowed a number of additional conclusions to be made, which have importance for the understanding of elafin biology generally. Firstly, the demonstration that all of elafin's anti-elastase activity could be accounted for by the COOH-terminus (Figure 2) provides supportive evidence for a single elastase inhibitory site in elafin, especially when considered in conjunction with the molecule's derived amino-acid sequence (Sallenave and Silva, 1993). However confirmation will only be forthcoming when the crystallographic structure of full-length elafin bound to HNE is available. It has previously been suggested that SLPI has a single inhibitory site for HNE but two reactive sites for combination with other enzymes such as cathepsin G (Boudier and Bieth, 1992). Secondly, the data in this chapter demonstrate antimicrobial effects for both the NH₂-terminus and COOH-terminus of elafin (Figure 5), adding to the known functional attributes of these domains outlined in section 1.5.5. The demonstration of antimicrobial activity but no anti-elastase activity for the NH₂-terminus of elafin (and unfolded elafin) indicates that most (and perhaps all) of elafin's antimicrobial effects are independent of anti-elastase activity.

It appears that elafin can be added to the array of endogenous cationic antimicrobial polypeptides expressed in man, even if elafin is a little larger than most of the molecules categorised in this way (Hancock, 1997). The prevailing view is that most cationic peptides exert their effect through interference with, and permeabilisation of, the bacterial cytoplasmic membrane (Hancock, 1997). Certainly PAO1 can be killed in this way (Sawyer et al., 1988). This raises the question of whether net charge alone can explain the effects of elafin described here.

The net charge of the NH₂-terminus of elafin is +5, while that of the COOH-terminus is +2. If an effect based entirely on net charge were to be invoked, then one might expect the effect of the NH₂-terminus to be intermediate between that of the COOH-terminus and the full-length molecule. This was not observed for either PAO1 or C1705 (Figures 5 and 8). Indeed the more positively charged NH₂-terminus appeared only to exert a marked antimicrobial activity when applied at high

concentrations to PAO1 (Figure 5). Furthermore, in the case of PAO1 an antimicrobial effect of HSA (which carries a net negative charge) was observed. On these grounds it seems most unlikely that the effects described here can be attributed solely to net charge. However a more subtle charge-related effect cannot be excluded at this stage, as it has been suggested that critical antimicrobial effects may be attributable to individual arginine residues, or to short positively charged fragments of amino acids within cationic peptides (Shafer et al., 1996).

While net charge appears not to be the sole determinant of elafin's antimicrobial activity, a number of limited inferences can be made from trends emerging in this chapter. For example for both PAO1 and C1705 the most effective concentrations of elafin (1 to 2.5 μ M, and 25 μ M respectively) were characterised by a greater effect of the whole molecule than the sum of its component parts (Figures 5 and 8). This could be interpreted as indicating a synergistic effect of the two domains, and possibly dual mechanisms of action. Curiously however, polypeptide folding did not appear to be critical, as for both organisms unfolded elafin exerted an antimicrobial effect, albeit weaker than that of the folded molecule. No firm conclusions should be drawn from these observations, but it is tempting to speculate that each of elafin's domains effect a separate antimicrobial function which is not crucially dependent on tertiary structure.

A further interesting point in assessing the antimicrobial activity of elafin is that the effects on PAO1 and C1705 appeared to be quite distinct (compare Figures 5 and 8). In particular the effect of elafin on PAO1 was maximal at low concentrations while the effect on C1705 was maximal at higher concentrations. Similarly, while high concentrations of the NH₂-terminus appeared to have a significant antimicrobial effect against PAO1, this moiety had minimal effect against C1705. Why elafin's effect on PAO1 should reveal an inverse dose-response relationship (Figure 3) is unclear. One potential explanation could be that *P. aeruginosa* uses elafin as nutrient, given the propensity of this organism to survive and grow in the presence of limited nutritional sources (Terry et al., 1991); a critical balance between elafin killing bacteria on one hand and providing a source of nutrition on the other may be tipped more in favour of killing at lower concentrations of elafin.

The emerging picture is of a complex interaction between elafin and components of individual bacteria or classes of bacteria. This degree of complexity is in keeping with recent observations made in relation to other antimicrobial

peptides. For example it is now recognised that cationic peptides may have diverse intracellular targets in addition to activity against cytoplasmic membranes (Xiong et al., 1999; Friedrich et al., 2000). Furthermore, it is now clear that cationic peptides can interact not only with important components of Gram-negative bacteria such as LPS (Scott et al. 2000), including that native to *P. aeruginosa* (Peterson et al., 1985), but also with critical structures of Gram-positive bacteria, such as teichoic acid in *S. aureus* (Scott et al., 1999). Additional complexity is afforded by the observation of synergistic activity for endogenous antimicrobial peptides, as has been demonstrated against *S. aureus* (Nagaoka et al., 2000). This field of research continues to expand, and it seems likely that the specific mechanisms responsible for elafin's antimicrobial effect will be complex.

When considering the antimicrobial role of elafin in the broader context of pulmonary defence, it is worthwhile first considering the known effects of elafin's "sister molecule" SLPI, and the interactions between bacteria and protease inhibitors generally.

SLPI has been shown to have activity against a wide variety of clinically important micro-organisms. These have included bacteria such as *Staphylococcus epidermidis* and *Escherichia coli* (Hiemstra et al., 1996; Wiedow et al., 1998), fungi such as *Candida albicans* and *Aspergillus fumigatus* (Tomee et al., 1997; Wiedow et al., 1998), and viruses such as Sendai virus, influenza A, and HIV-1 (McNeely et al., 1995; McNeely et al., 1997; Kido et al., 1999). In the specific context of this study, SLPI has been shown to have activity against *S. aureus* (Hiemstra et al., 1996) and *P. aeruginosa* (Wiedow et al., 1998) and to bind LPS from *P. aeruginosa* (Ding et al., 1999). The antimicrobial activity of SLPI against *S. aureus* and *P. aeruginosa* in comparable studies (Hiemstra et al., 1996; Wiedow et al., 1998) was more profound than that described here (Figures 6 and 10). Interestingly, the studies of Hiemstra et al. (1996) showed that the antimicrobial effects of SLPI were independent of the molecule's anti-elastase activity, but in contrast to the findings described here for elafin, they also described a profound effect for the structural NH₂-terminus against *S. aureus*. The lower levels of antimicrobial activity described for SLPI and the relative inactivity of the NH₂-terminus described in this work may reflect the different strains of *P. aeruginosa* and *S. aureus* used. Both the considerable homology between SLPI and elafin, and the broadly similar antimicrobial activities described in this chapter (Figures 6 and 10) raise speculation as to whether elafin may have similarly diverse patterns of antimicrobial activity. In this regard it is

interesting that SLPI and elafin did not display synergistic activity against *S. aureus* or *P. aeruginosa*, especially when significant synergistic interactions against *E. coli* have been demonstrated for the combination of SLPI, lysozyme and lactoferrin (Singh et al., 2000).

Although the work described by Hiemstra et al. (1996) in relation to SLPI and the results described here in relation to elafin clearly dissociate antimicrobial activity from anti-elastase activity, this is not to say that bacteria cannot influence the function of anti-elastases. It is known that elastase produced by *P. aeruginosa* and protease produced by *S. aureus* can inactivate SLPI and α_1 -PI, though interestingly SLPI seemed considerably less susceptible to proteolytic degradation (Sponer et al., 1991). Extending these observations, alginate, a characteristic product of mucoid *P. aeruginosa*, has been shown to accelerate binding of SLPI to HNE but to inhibit association of HNE and α_1 -PI (Ying et al., 1996). These general principles can be observed in other cationic antimicrobial peptides, in that defensins can block the inhibition of HNE by α_1 -PI (Panyutich et al., 1995). In addition, whilst no direct evidence exists to demonstrate an influence of proteases and antiproteases in the adhesion of *S. aureus* to host tissues, it is intriguing that elastin-binding protein should be considered an important determinant of Staphylococcal colonisation (Park et al., 1991). It is likely therefore that the complex interaction between elafin and bacteria described here are still more complex in the infected and inflamed respiratory tract.

SLPI and elafin therefore appear to join an impressive array of endogenous human antimicrobial polypeptides, many of which are detectable in the lung. Perhaps the best characterised of the endogenous pulmonary antimicrobial agents are the defensins, which also exhibit a broad range of antimicrobial activity against Gram-positive and Gram-negative pathogens, in addition to antiviral and antifungal functions (Ganz et al., 1985; Lehrer et al., 1993). The inextricable link between defensins and anti-elastases touched upon in Chapter 3 is thus reinforced in this chapter, hinting at dynamic interactions between these groups of molecules (van Wetering et al., 2000). Among the other endogenous cationic antimicrobial peptides potentially liberated into human airways are lactoferrin and lysozyme (Ellison and Giehl, 1991), cathelicidins including LL-37/human CAP-18 (Bals et al., 1998), and BPI (Elsbach and Weiss, 1993). The relative contribution of these endogenous peptides in health and during pulmonary inflammation has not yet been elucidated. However it is interesting that, in a single study of patients free from infection and

having bronchoscopy for investigation of suspected bronchial carcinoma, the most prominent antimicrobial agents in BALF were lysozyme and human neutrophil defensins 1, 2 and 3 (Schnapp and Harris, 1998).

Several features of endogenous cationic antimicrobial peptides make them attractive for therapeutic development. These include a broad-spectrum of antimicrobial activity for most cationic peptides (Lehrer et al., 1993; Turner et al., 1998), and a tendency for synergy with conventional antibiotics (Giacometti et al., 2000), presumably as a consequence of increased permeability of the cytoplasmic membrane (Hancock, 1997). Additional attractions include the ability to clear organisms associated with high levels of resistance to conventional antibiotics, as has been demonstrated here for PAO1 and by others (Turner et al., 1998), and the theoretical potential for reduced emergence of resistance to cationic peptides when used in a therapeutic context (Hancock, 1997).

However cationic peptides could also present unique problems, dictating that further characterisation of these molecules is required before their application is broadened significantly. These problems include potential cytotoxicity when applied to mammalian cells as has already been discussed in the context of the defensins (Lichtenstein et al., 1986), the recognition of inherent resistance to cationic peptides in certain human pathogens (Groisman, 1994; Bayer et al., 2000; MacFarlane et al., 2000; Ganz et al., 2001; Peschel et al., 2001), and (specifically in the context of CF) the ongoing debate as to whether cationic peptides are biologically active in the presence of high salt concentrations (Smith et al., 1996; Goldman et al., 1997b; Nagaoka et al., 2000; Travis et al., 2000).

5.4. SUMMARY

The data in this chapter indicate that elafin has antimicrobial activity against Gram-positive and Gram-negative respiratory pathogens. These findings add weight to the theory that elafin may play an important role in innate immunity, being a rapidly inducible antimicrobial agent expressed at epithelial surfaces, with the potential to recruit neutrophils whilst protecting tissue against extracellular HNE and proteinase 3.

This combination of properties, allied to the demonstration of efficient elafin gene transfer to the murine lung, suggested that appropriate criteria prevailed to test the hypothesis that genetic augmentation may confer protection in an animal model of acute inflammatory lung injury.

CHAPTER 6

THE EFFECT OF ELAFIN GENE AUGMENTATION ON ACUTE LUNG INJURY MEDIATED BY *PSEUDOMONAS AERUGINOSA*

6.1. AIMS

The aim of the work in this chapter was to determine whether genetic augmentation of human elafin could protect lung tissue in an animal model of acute inflammatory injury. Experiments described in previous chapters demonstrated that elafin augmentation was feasible using IT administration of Ad-elafin. They also suggested that elafin may have an important role in early innate immunity. For a number of reasons it seemed appropriate to study the effects of elafin augmentation on acute lung injury induced by *P. aeruginosa*. For example, murine pneumonia is readily induced by *P. aeruginosa*, novel strategies for elimination of this bacterium are required on account of its pathogenicity and antibiotic resistance, and elafin has *in vitro* effects both against the organism itself and against activated neutrophils.

6.2. RESULTS

6.2.1. Bacterial growth and MPO activity in lungs from normal C57/Bl6 mice.

The total cell count, differential cell count and protein concentration of BALF from normal (untreated) mice were established in section 4.2.1., and act as normal values for cross-reference. Extending these themes to the context of this chapter, 4 normal mice were sacrificed for retrieval of BALF and lungs. BALF and homogenised right lung from each of the mice was plated onto PIA without dilution and no growth was detected. Left lungs were weighed and homogenised, and MPO activity estimated. The median MPO activity in undiluted homogenate (assessed by adding MPO-specific substrate and measuring change in absorbance at 630nm as a function of time) was 84 absorbance units/minute. When corrected for weight the median level was 0.88 absorbance units/minute/mg.

6.2.2. Preliminary experiments to determine the effects of *P. aeruginosa* on murine lungs.

Preliminary experiments were performed to characterise the pulmonary damage induced in murine lungs after IT administration of *P. aeruginosa*, and to determine total lung MPO activity as a surrogate for neutrophil accumulation (Figure 1). All mice tolerated the procedure well. Administration of *P. aeruginosa* resulted in a dose-dependent increase in BALF protein concentrations (taken to reflect disruption of the alveolar-capillary membrane) (Figure 1). While protein concentrations using the highest dose of *P. aeruginosa* were considerably above normal at both time points analysed, injury appeared to be more pronounced at 4 hours than at 24 hours (Figure 1). In keeping with these findings bacteria were isolated from lung in a dose-dependent fashion at both time points, many more bacteria being isolated at 4 hours (Figure 1). However there was considerable variability in the number of bacteria retrieved from individual mice.

MPO activity in homogenised lung approximated to normal levels in mice receiving the lower dose of *P. aeruginosa* but showed an apparently sustained elevation in mice receiving the higher concentration of bacteria (Figure 1).

It must be emphasised that a number of technical problems accompanied this initial, preliminary experiment, and the results should be interpreted cautiously. For example the initial dose of *P. aeruginosa* was suggested on the basis of historical experience in the Department of Microbiology. The intention had been to produce bacterial growth consistently at 24 hours, but this did not occur in this experiment. Secondly, while the 24 hour time point was strictly observed, technical considerations relating to the checking of absorbance of bacterial suspensions, and the variable time taken for anaesthesia/dosing/repeat anaesthesia/organ retrieval meant that "4 hours" in fact represented between 4 and 6 hours in practice. Thirdly, lung MPO levels were not corrected for lung weight in this initial experiment. Finally small numbers were studied, and thus comparison between groups is made difficult by the inherent variability in bacterial counts.

Despite these experimental limitations, it seemed reasonable to make the cautious conclusion that IT *P. aeruginosa* appears to be attended by rapid neutrophil accumulation in the lung, and that the inflammatory process damages the alveolar-capillary membrane. There was also a strong suggestion that mice have the intrinsic

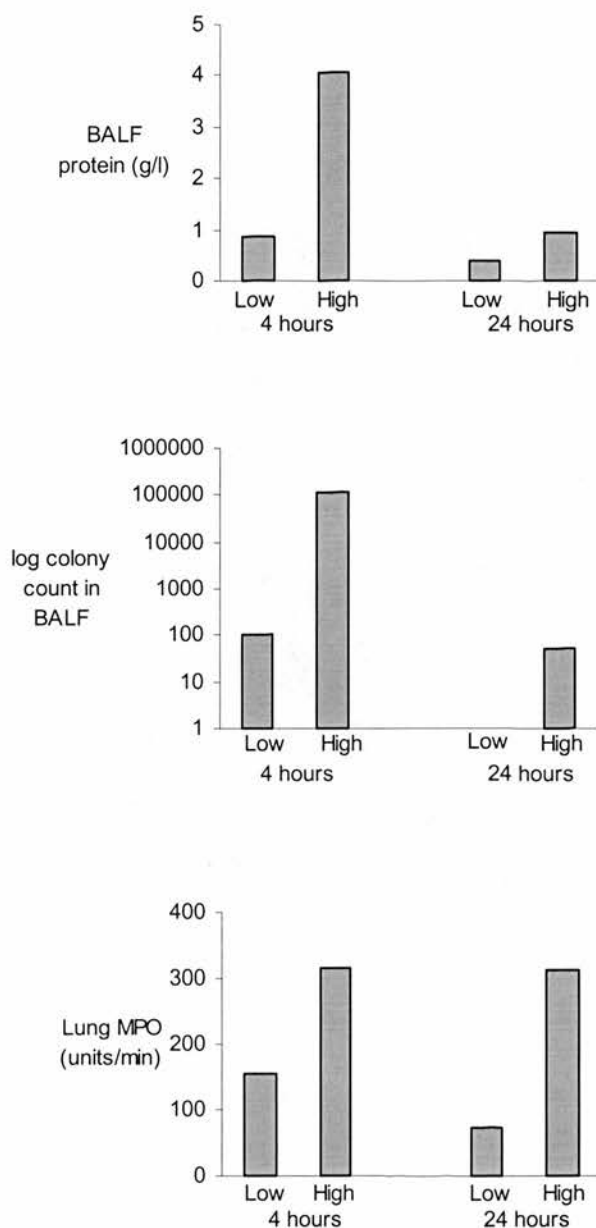


Figure 1. *P. aeruginosa* delivered IT is associated with lung injury in mice.

A suspension of *P. aeruginosa* PAO1 was prepared with an absorbance at 590 nm of 1.42. The suspension was diluted 8-fold ('high dose') and 800-fold ('low dose') in 0.01M phosphate buffer. High dose or low dose was given IT to mice (40µl instillations, n=6 in each group). From each group 3 mice were killed at 4 hours and 3 at 24 hours. Protein concentration and colony count (on PIA) were determined in BALF. MPO was measured in lung homogenates. Results are expressed as medians.

capacity to clear *P. aeruginosa* rapidly.

On the basis of these findings a second preliminary experiment was performed in which 5 mice received 40µl of IT *P. aeruginosa* suspension (absorbance at 590nm of 1.45) and were killed 4 hours later. Median protein concentration in BALF was 2.1 g/l (range 0.7 - 3.8 g/l) and MPO activity in undiluted lung homogenates (corrected for lung weight) were 3.25 absorbance units/minute/mg. BALF and homogenised lung were diluted 100-fold in phosphate buffer and applied to PIA overnight at 37°C. In 4 of the mice a lawn of bacteria emerged precluding accurate colony counting. A few scattered colonies were found in undiluted whole blood (range 0-17 colonies) and undiluted splenic homogenates (range 2-8 colonies) applied to PIA. In the fifth mouse no colonies were found in homogenised lung, and 7 colonies were found in undiluted BALF. Interestingly the blood from this mouse yielded 42 colonies.

Together these experiments illustrated the capacity for *P. aeruginosa* to injure lung tissue, but also exemplified technical difficulties and the variability in response of individual mice.

Before proceeding to study whether Ad-elafin could protect the murine lung against the effects of *P. aeruginosa*, it was also necessary to determine whether adenoviral transfection altered the susceptibility of mice to "background" bacterial infection/colonisation. Mice therefore received IT Ad-elafin (3×10^7 pfu), Ad-lacZ (3×10^7 pfu) or PBS (n=4 in each group), followed 5 days later by IT administration of PBS. The mice tolerated the procedure well and were killed 4 hours later. Undiluted whole blood, homogenised spleen, homogenised lung and BALF were plated onto PIA and no growth was detected in samples from any of the mice, thus excluding propensity to colonisation with *P. aeruginosa*. Elafin was detectable in BALF from all Ad-elafin mice (median 3.6 ng/ml) and undetectable in BALF from control mice.

With these preliminary observations in place, experiments were performed to establish the effect of Ad-elafin on lung injury mediated by *P. aeruginosa* at 4 hours and 24 hours.

6.2.3. The effect of Ad-elafin on lung injury induced by *P. aeruginosa* at 4 hours.

Mice received IT Ad-elafin (3×10^7 pfu), Ad-lacZ (3×10^7 pfu) or PBS (n=4 in each group), followed 5 days later by IT administration of *P. aeruginosa* PAO1 (40µl of a suspension with an absorbance at 590nm of 1.42). The mice were killed 4 hours later, with retrieval of BALF and lungs. Interestingly, the highest bacterial counts were retrieved from Ad-lacZ treated mice, while a relative pulmonary neutrophilia was found both in Ad-lacZ and Ad-elafin treated mice (Table 1). In contrast, all groups had a modest elevation in BALF protein concentration, this being least pronounced in the Ad-lacZ treated animals. Median elafin concentration in BALF from Ad-elafin mice was 7.5 ng/ml ($p < 0.001$ when compared to mice receiving PBS for 4 hours on day 5 as opposed to receiving *P. aeruginosa* for 4 hours).

The small groups studied precluded detailed conclusions, but it appeared that Ad-elafin had no obvious protective effect in the first 4 hours after exposure to *P. aeruginosa*. However technical difficulties continued to make adherence to the 4 hour time point difficult. This problem was compounded by the fact that inherent variability in the biological responses of mice suggested that larger groups of mice were required to observe meaningful differences. These considerations (along with the experience gained in studying mice 24 hours after exposure to LPS as described in Chapter 4) led to more detailed examination of the effect of elafin gene augmentation on lung injury mediated by *P. aeruginosa* at 24 hours.

6.2.4. The effect of Ad-elafin on lung injury induced by *P. aeruginosa* at 24 hours.

The protocol designed for this set of experiments is summarised in Table 2. The original intention was to study 20 mice in each group. However 2 mice died immediately after the first anaesthesia. Two further mice (treated with Ad-elafin) died immediately after anaesthesia on day 5. All of these mice appeared healthy and well prior to anaesthesia. Finally, in two mice BALF could not be retrieved on account of a leak of fluid from the lung caused by surgical trauma. Both death under anaesthesia and surgical trauma to the lungs on dissection of the heart, lungs and trachea from the thoracic cage are occasional complications of the procedure described. Full information (as outlined in Table 2) was obtained for the remaining 54 mice.

	Total cell count in BALF	Neutrophil count in BALF	Macrophage count in BALF	MPO activity in lung (units/min)	Protein in BALF (g/l)	BALF PAOI colony count (cfu/ml)	Lung PAOI colony count (cfu/ml)
PBS	488000	205100	235200	309	1.01	224000	300000
Ad-lacZ	674000	438500	107800	374	0.77	710000	77000000
Ad-elafin	646000	498400	120700	439	1.00	246000	862000

Table 1. The effect of Ad-elafin on injury mediated by IT *P. aeruginosa* at 4 hours.

Mice received a 40µl IT instillation of Ad-lacZ (3×10^7 pfu), Ad-elafin (3×10^7 pfu) or PBS (n=4 in each group). Five days later all mice received a 40µl IT instillation of a suspension of *P. aeruginosa* PAOI with an absorbance at 590nm of 1.42. All mice were killed 4 hours later. BALF and lungs were retrieved. Measurements performed on BALF included total and differential cell count, bacterial colony count (on PIA) and protein concentration. Bacterial colony count (on PIA) and MPO activity were also established in homogenised lung. Results are expressed as medians.

n	DAY 0	DAY 5	DAY 6
20	PBS	<i>P. aeruginosa</i>	Mice killed with retrieval of.... SERUM for PAO1 colony count. SPLEEN for PAO1 colony count.
20	Ad-lacZ	<i>P. aeruginosa</i>	BALF for total cell count, neutrophil count, macrophage count; PAO1 colony count; concentrations of protein, albumin, elafin, MPO, EIA, mKC. LUNGS for PAO1 colony count, MPO, histology.
20	Ad-elafin	<i>P. aeruginosa</i>	

Table 2. Outline of experiments to determine the effect of Ad-elafin transfection on lung injury mediated by *P. aeruginosa* at 24 hours in C57/Bl6 mice.

C57/Bl6 mice received an IT instillation (40µl) of PBS or Ad-lacZ (3x10⁷ pfu) or Ad-elafin (3x10⁷ pfu). Five days later, the mice in each group received IT *P. aeruginosa* (34 µl). Twenty-four hours later mice were killed. Serum, spleen, BALF and lungs were retrieved.

Four aliquots of the bacterial suspension (absorbance at 590nm of 1.42-1.44) prepared were plated on PIA immediately prior to dosing, and incubated overnight at 37°C. The mean colony count was 2.2×10^{11} cfu/ml.

The principal aim of this set of experiments was to determine whether Ad-elafin protected the lung against acute inflammatory injury, as assessed by protein concentrations measured in BALF. Ad-elafin was associated with a significant reduction in acute lung injury (Figure 2). There was, none the less, considerable variability in protein concentrations (as evidenced by the error bars in Figure 2), and it was certainly not the case that all Ad-elafin treated mice were protected as compared with controls. In assessing the degree of protection conferred, it is necessary to recall the "normal" protein concentration in BALF, the median value calculated in Chapter 4 being 0.45 g/l. The average degree of protection conferred in this experiment therefore approximates to

$$[(\text{median BALF protein concentration in control mice} - 0.45 \text{ g/l}) - (\text{median BALF protein concentration in Ad-elafin mice} - 0.45 \text{ g/l})] \div (\text{median BALF protein concentration in control mice} - 0.45 \text{ g/l})$$

Thus the average reduction in acute lung injury relative to Ad-lacZ mice was approximately 43% and the average reduction relative to PBS mice was approximately 46%.

A similar trend was observed when studying murine albumin concentrations in BALF (Figure 3). The average concentration in Ad-elafin treated mice was significantly lower than that in mice treated with PBS, with Ad-lacZ treated mice having an intermediate level. Variability in BALF albumin levels was high, emphasising the wide range of responses within each group of mice.

The BALF retrieved from control mice generally appeared haemorrhagic in comparison with that from Ad-elafin mice. Histological appearances also suggested less severe damage in Ad-elafin treated animals (Figure 4). Damage occurred in all lobes of the lung, suggesting widespread distribution of the bacterial suspension. Taking these strands of information together it appeared that control mice were subject to a diffuse bronchopneumonia, characterised by pulmonary neutrophilia and alveolar haemorrhage (Figure 4).

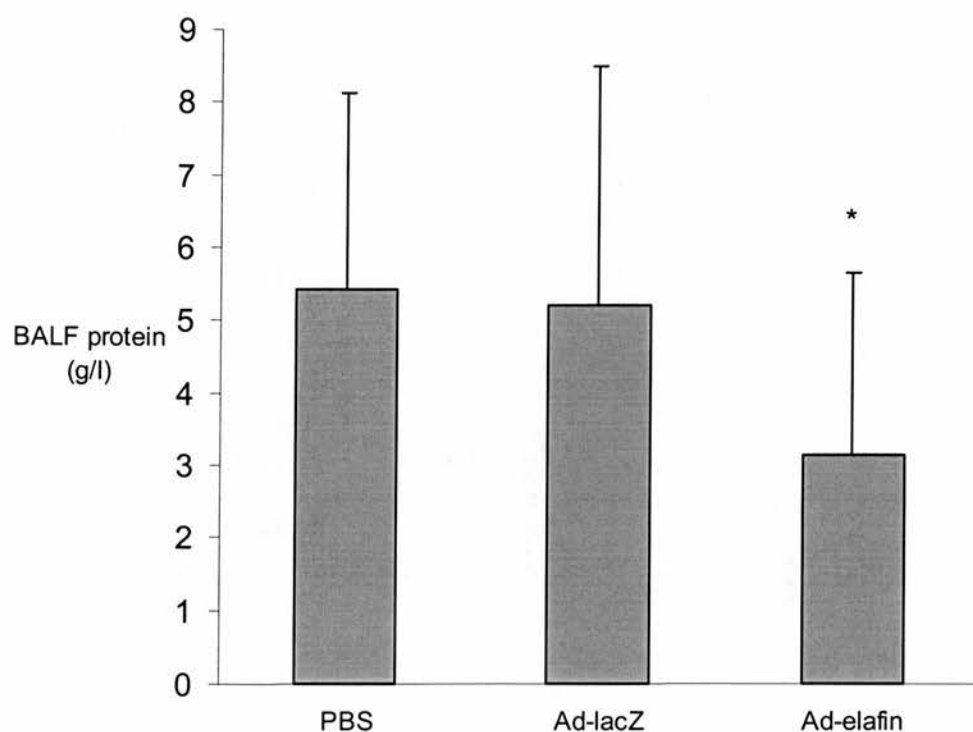


Figure 2. Ad-elafin significantly protects the murine lung against injury mediated by *P. aeruginosa*.

Mice were treated as described in Table 2. Protein concentration was measured in BALF.

For the PBS group n=18, for the Ad-lacZ group n=20, for the Ad-elafin group n=16. Results are expressed as means and standard deviations.

* = significant difference, $p < 0.05$ when compared with each of the other groups.

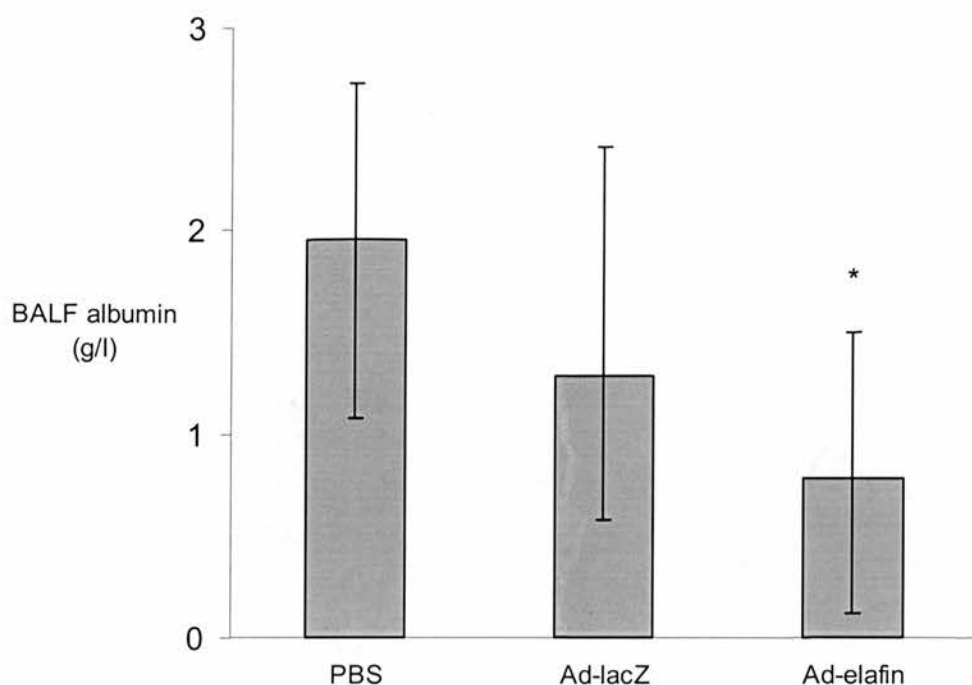


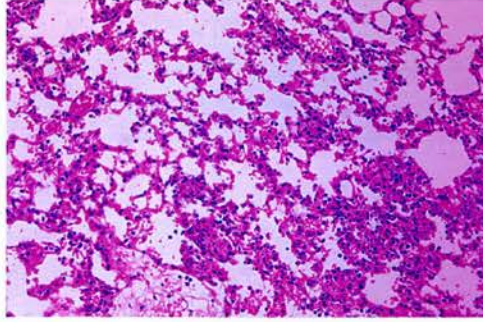
Figure 3. Ad-elafin administration results in a relative reduction in BALF albumin concentrations provoked by IT *P. aeruginosa*.

Mice were treated as described in Table 2. Albumin concentration was measured in BALF.

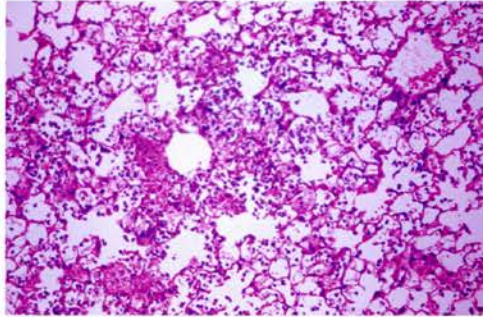
For the PBS group n=18, for the Ad-lacZ group n=20, for the Ad-elafin group n=16. Results are expressed as medians and interquartile ranges.

* = significant difference, $p < 0.05$ when compared with the PBS group.

A



B



C

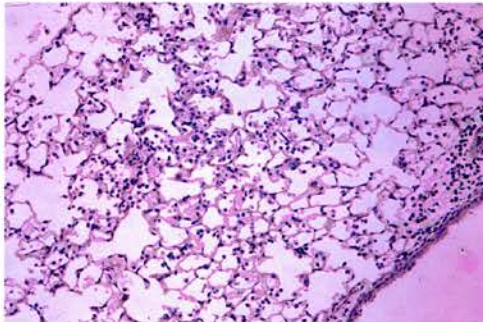


Figure 4. Ad-elafin protects the lung against injury mediated by *P.aeruginosa*: histology.

Mice were treated as described in Table 2. Lungs were removed, fixed in paraffin, sectioned and stained with haematoxylin and eosin. Histological sections from representative mice are shown. Marked protection is conferred by Ad-elafin.

PANEL A: IT PBS followed by IT *P. aeruginosa*.

PANEL B: IT Ad-lacZ followed by IT *P. aeruginosa*.

PANEL C: IT Ad-elafin followed by IT *P. aeruginosa* (original magnification x 40 in each case).

The Ad-elafin treated mice had a median elafin concentration in BALF of 8.5 ng/ml (interquartile range 5.9-10.4 ng/ml). Fifteen of the 16 mice had detectable levels of elafin. None of the control mice had detectable human elafin.

The protection associated with elafin was accompanied by a significant reduction in the number of *P. aeruginosa* colonies isolated from BALF (Figure 5). The average number of colonies retrieved from the BALF of Ad-lacZ mice was considerably lower than the number from PBS mice, but the difference did not achieve statistical significance (Figure 5). With regard to the effect of Ad-elafin a similar pattern was found when analysing the number of *P. aeruginosa* colonies in whole lung homogenates there being, on average, markedly fewer colonies in lungs from Ad-elafin treated mice (Figure 6). The differences noted in lung homogenates approached, but did not reach, statistical significance ($p=0.08$ when comparing Ad-elafin and Ad-lacZ mice, $p=0.07$ when comparing Ad-elafin and PBS mice). It should be emphasised that the ordinate describes a logarithmic scale in Figures 5 and 6. Among Ad-elafin mice there was a significant negative correlation between the elafin concentration in BALF and the (log transformed) *P. aeruginosa* colony count in both BALF ($r=-0.75$, $p<0.05$), and lung ($r=-0.85$, $p<0.01$).

The trend for more effective elimination of bacteria in Ad-elafin treated mice was also observed in whole blood and splenic homogenates applied to PIA. Colonies were detectable in the blood of 31% of Ad-elafin mice as opposed to 44% of Ad-lacZ mice and 61% of PBS mice. Similarly colonies were detectable in splenic homogenates from 19% of Ad-elafin mice in contrast to 40% of Ad-lacZ mice and 44% of PBS mice.

With regard to the cellular content of BALF, no significant difference was found in the total cell count or neutrophil count when comparing the 3 groups of mice (Figures 7 and 8). There was a trend for virus-treated mice to have lower cell counts than PBS treated animals, but this did not attain statistical significance.

In contrast Ad-elafin treatment was associated with a trend towards lower levels of neutrophil recruitment to the lung as manifest by a lower level of MPO activity in lung homogenates (Figure 9). While the available volumes of BALF limited the opportunities to measure multiple neutrophil chemokines, the level of mKC was kindly measured by Professor T. Standiford and colleagues (Ann Arbor, MI, USA) and found to be lowest in Ad-elafin treated mice (median level 0.14 ng/ml

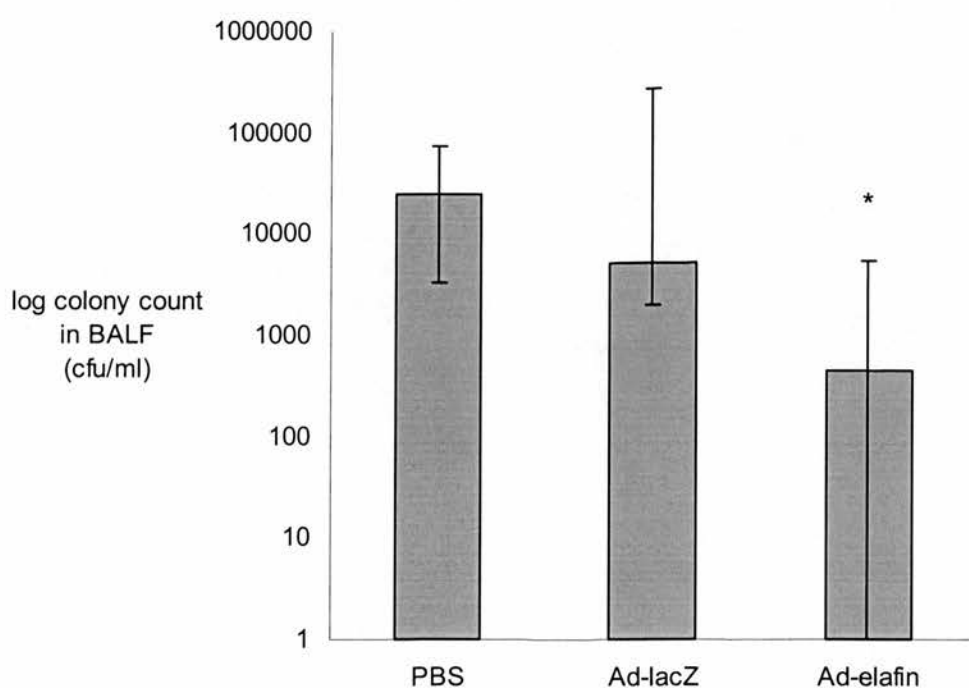


Figure 5. Ad-elafin administration significantly increases clearance of *P. aeruginosa* from BALF.

Mice were treated as described in Table 2. *P. aeruginosa* colony counts were established in BALF.

For the PBS group n=18, for the Ad-lacZ group n=20, for the Ad-elafin group n=16.

Results are expressed as medians and interquartile ranges.

* = significant difference, $p < 0.05$ when compared with each of the other groups.

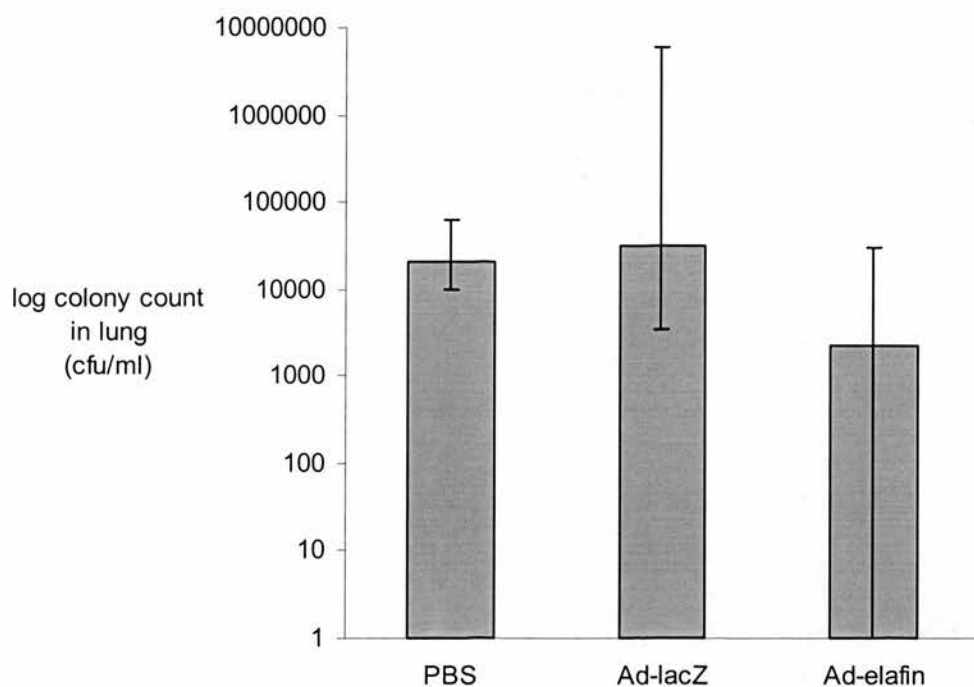


Figure 6. Ad-elafin administration is associated with increased clearance of *P. aeruginosa* from lung.

Mice were treated as described in Table 2. *P. aeruginosa* colony counts were established in homogenised lung.

For the PBS group n=20, for the Ad-lacZ group n=20, for the Ad-elafin group n=16.

Results are expressed as medians and interquartile ranges.

No statistically significant differences were observed when comparing groups.

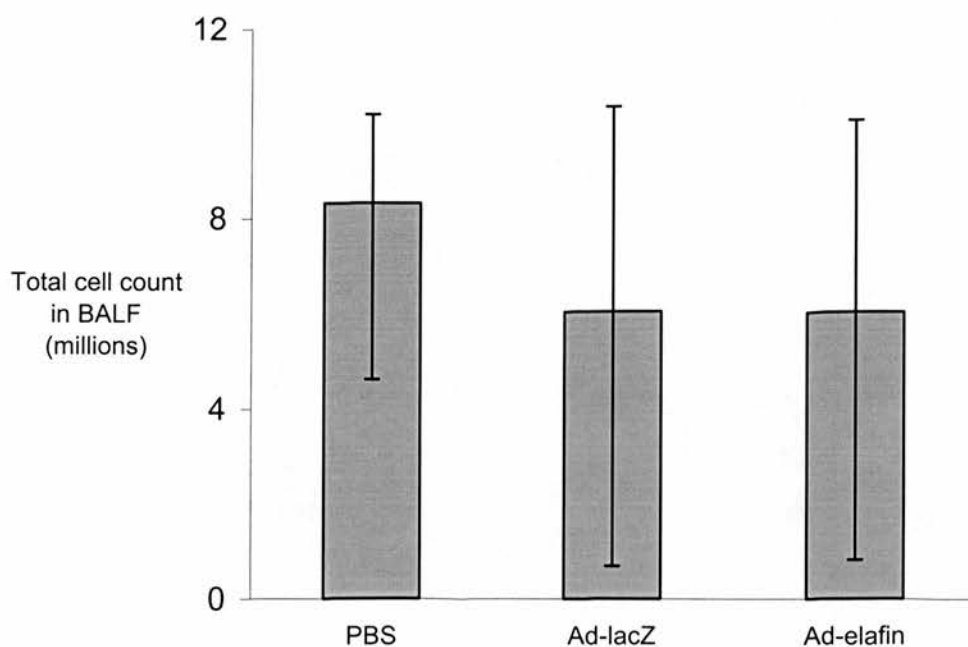


Figure 7. Total cell count in BALF from mice receiving IT *P. aeruginosa*.

Mice were treated as described in Table 2. The total cell count in BALF was established.

For the PBS group n=18, for the Ad-lacZ group n=20, for the Ad-elafin group n=16.

Results are expressed as medians and interquartile ranges.

No significant differences were observed when comparing groups.

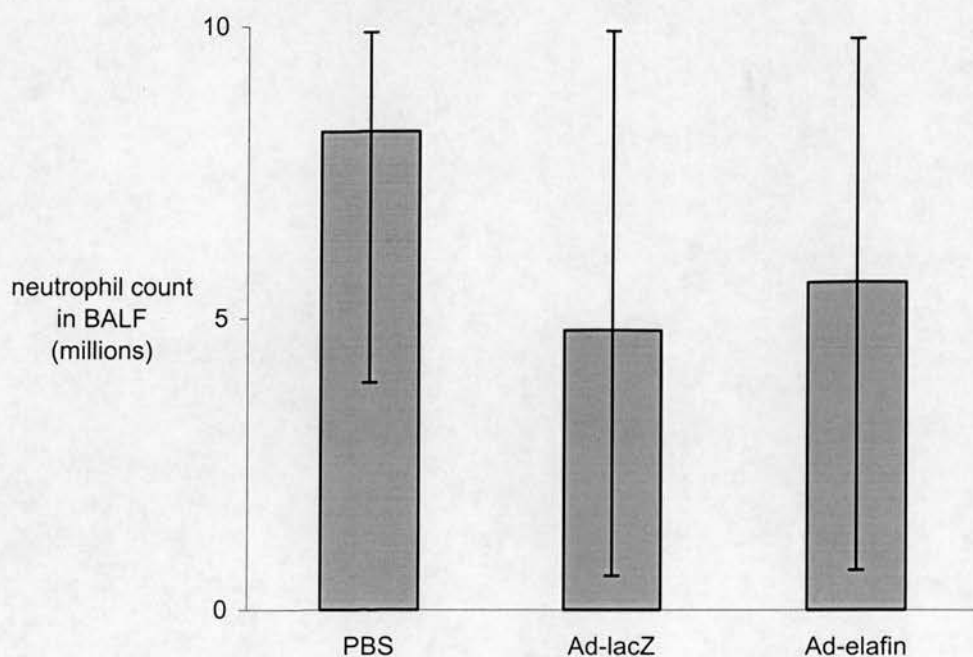


Figure 8. Neutrophil count in BALF from mice receiving IT *P. aeruginosa*.

Mice were treated as described in Table 2. The neutrophil count in BALF was established.

For the PBS group n=17, for the Ad-lacZ group n=19, for the Ad-elafin group n=16. Results are expressed as medians and interquartile ranges.

No significant differences were observed when comparing groups.

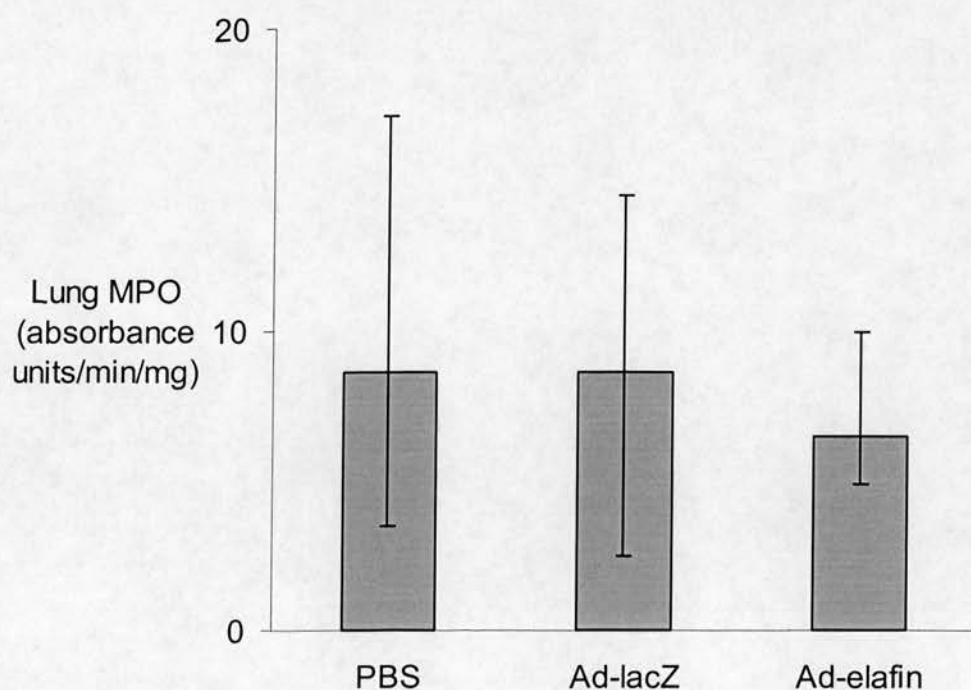


Figure 9. MPO activity in homogenised lungs from mice receiving IT *P. aeruginosa*.

Mice were treated as described in Table 2. The MPO level in homogenised lung was established.

For the PBS group n=20, for the Ad-lacZ group n=17, for the Ad-elafin group n=14.

Results are expressed as medians and interquartile ranges.

No significant differences were observed when comparing groups.

in Ad-elafin mice, 0.34 ng/ml in Ad-lacZ mice, and 0.25 ng/ml in PBS mice; significant difference comparing Ad-elafin and Ad-lacZ mice, $p<0.05$). Similarly, there was a suggestion that Ad-elafin treatment was associated with inhibition of neutrophil degranulation in that there was less MPO detectable in BALF from Ad-elafin mice (Figure 10). While the trend in all these neutrophil-related parameters was towards a reduction among Ad-elafin mice, it must be stated that the only statistically significant difference was as described for mKC comparisons. In keeping with all parameters measured in these experiments, there was considerable variation in the values obtained from mice within the same group (as reflected in the error bars in Figures 9 and 10).

In contrast, a different pattern was observed when considering macrophage counts in BALF. Median macrophage count (and interquartile range) was 300000 (150000-510000) for PBS treated mice, 220000 (120000-460000) for Ad-lacZ treated mice and 310000 (170000-560000) for Ad-elafin treated mice (no significant difference between groups).

Despite the striking influx of neutrophils to airways and lung parenchyma in each of the groups studied, no mouse was found to have spontaneous elastase activity in BALF. As such a relative excess of elastase inhibitors prevailed in all mice. The average EIA was in the low μM range for all groups studied, but was lower in the Ad-elafin mice than in either of the control groups, the difference becoming statistically significant when comparing Ad-elafin and PBS mice (Figure 11). In the Ad-elafin mice, human elafin appears to have contributed less than 1% of the measured EIA. This observation is based on the following assumptions: if it is assumed that the elafin produced has a molecular mass of around 10 kDa, then the average elafin concentration in BALF of 8.5 ng/ml predicts for a concentration of 0.85 nM. If all of the elafin were assumed to be active (and given that the average EIA in BALF from Ad-elafin mice was 0.73 μM) then the contribution of human elafin to total EIA would be approximately $(0.85 \text{ nM} \times 100) \div 730 \text{ nM} = 0.12\%$.

A strong correlation was found between EIA and protein concentration in BALF, suggesting that EIA may have developed in response to injury in this model (Figure 12). Protein concentration in BALF also correlated with other markers of lung injury (for albumin concentration $r=0.87$, $p<0.0001$), with bacterial indices (for bacteria in BALF $r=0.67$, $p<0.0001$; for bacteria in lung $r=0.60$, $p<0.0001$), and with neutrophil indices (for neutrophil count in BALF $r=0.51$, $p=0.0003$; for MPO in

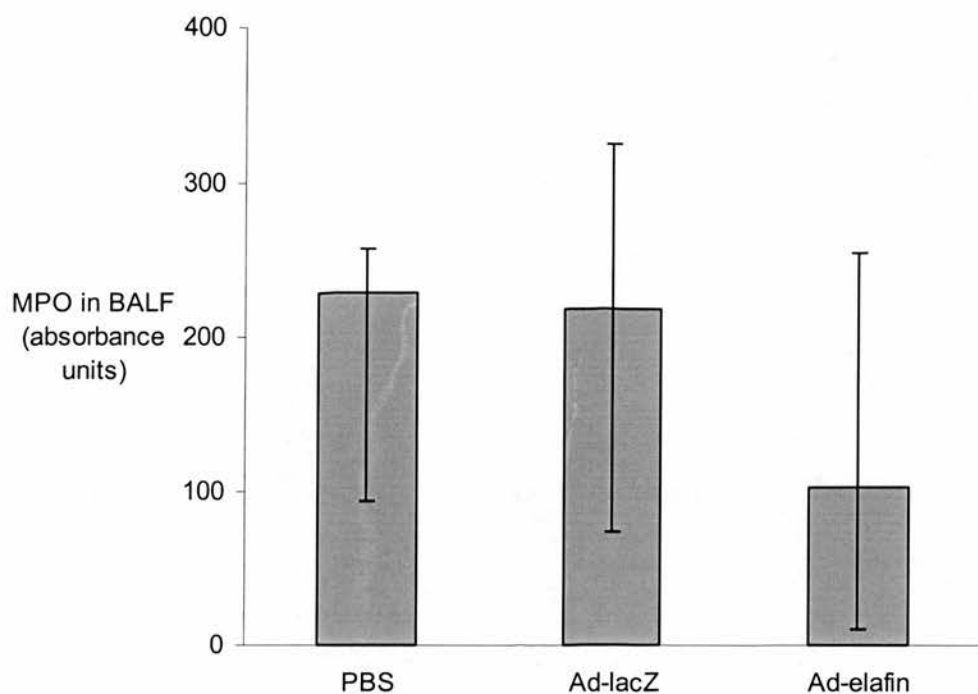


Figure 10. MPO activity in BALF from mice receiving IT *P. aeruginosa*.

Mice were treated as described in Table 2. The MPO activity in BALF was established.

For the PBS group n=17, for the Ad-lacZ group n=20, for the Ad-elafin group n=16.

Results are expressed as medians and interquartile ranges.

No significant differences were found when comparing groups.

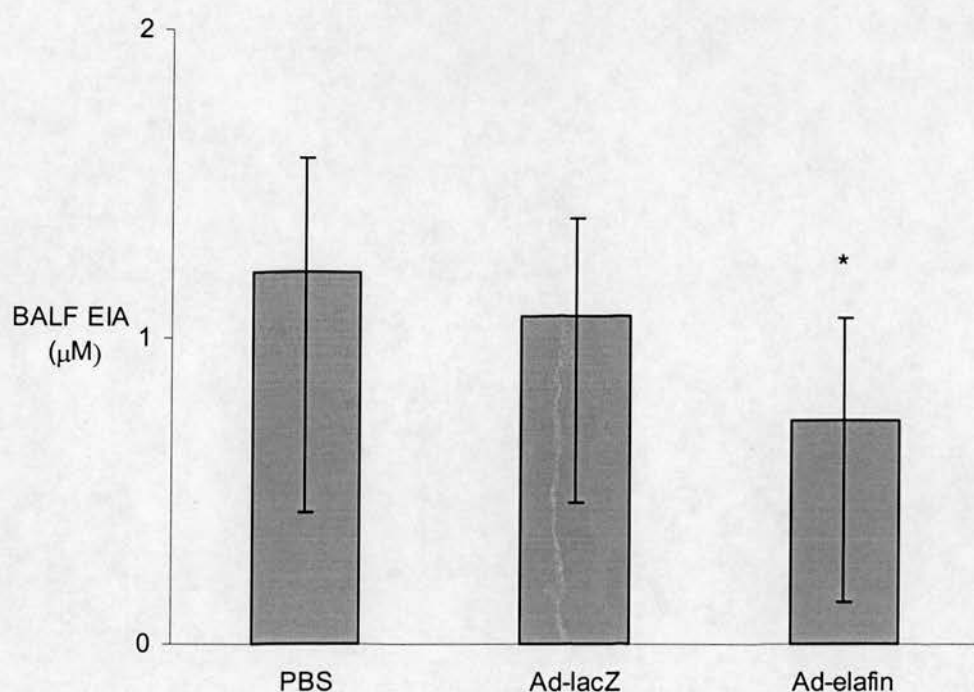


Figure 11. Ad-elafin administration is associated with a relative reduction in the elastase inhibitory activity of BALF from mice treated with IT *P. aeruginosa*.

Mice were treated as described in Table 2. EIA was measured in BALF.

For the PBS group n=18, for the Ad-lacZ group n=20, for the Ad-elafin group n=16.

Results are expressed as medians and interquartile ranges.

* = significant difference, $p < 0.05$ when compared with the PBS group.

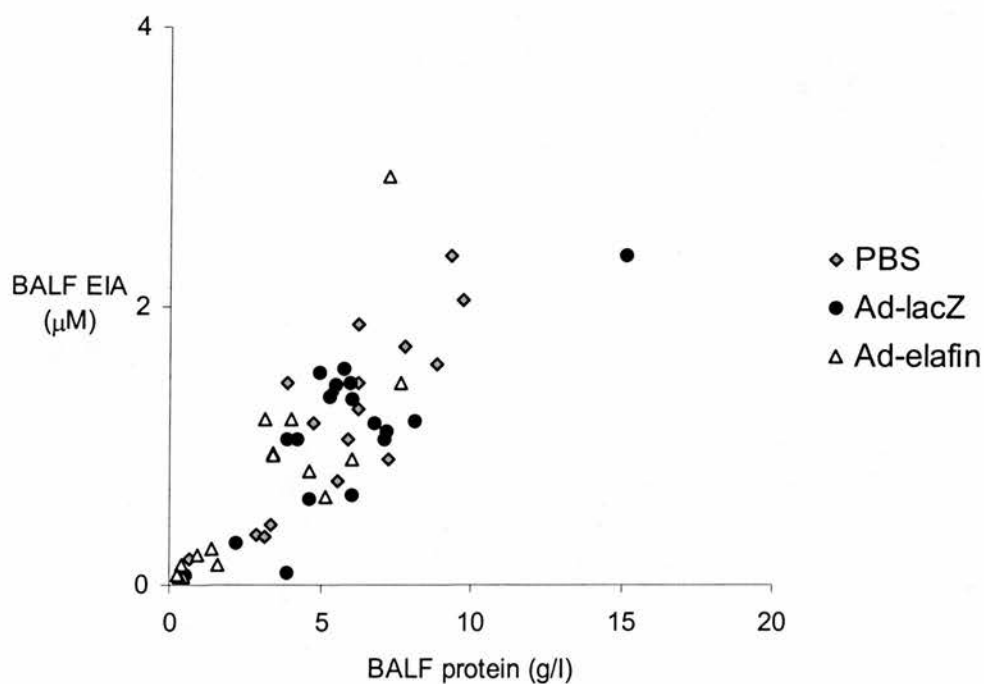


Figure 12. EIA and protein concentration are closely correlated in BALF after injury with IT *P.aeruginosa*.

Mice were treated as described in Table 2. EIA and protein concentration were measured in BALF.

The correlation described is statistically significant ($r=0.82$; $p=0.0001$).

BALF $r=0.75$, $p<0.0001$). No significant correlation was found between protein concentration and macrophage count in BALF.

6.3. DISCUSSION

The principal finding in this chapter relates to the significant tissue protection conferred by Ad-elafin in an animal model of acute inflammatory lung injury. Discussion of these data will focus on the model used to initiate lung injury, on the role of elafin (and adenovirus) in effecting protection, and on the emerging potential for gene therapy strategies in models of pulmonary infection.

6.3.1. Lung injury induced by *P. aeruginosa* PAO1.

Lung injury induced by *P. aeruginosa* is well described and has for example been demonstrated using strain PAO1 (George et al., 1993). Two broad categories of model exist, the first involving IT instillation of a slurry of *P. aeruginosa* laden microscopic beads, and the second involving IT instillation of a bacterial suspension in the absence of beads. In general the peak of lung injury is produced later in the bead model (as for example in van Heeckeren et al., 2000). It should be noted that murine models of *P. aeruginosa* lung injury described in the literature commonly use different bacterial strains and are applied to a variety of different strains of mouse, so difficulties exist in attempting to extrapolate from one study to another.

In this study damage was readily induced in the lungs of mice 24 hours after IT instillation of *P. aeruginosa* as reflected in the haemorrhagic appearance of BALF and histological appearances of lungs from control mice (Figure 4). Indeed the bronchopneumonia induced in this study was typical of that described elsewhere (as for example in Gosselin et al., 1998). The principal end-point used to assess alveolar epithelial injury in this study was the protein concentration in BALF. This measurement is widely used as a marker of lung injury, and increased concentrations are thought to represent significant leak across a disrupted alveolar-capillary membrane (reviewed by Pittet et al., 1997). The technique is simple and reproducible, and unlike other methods for measuring alveolar-capillary leak avoids the requirement for systemic infusion of, for example, radioactivity or marker dye. Potential problems in measuring protein concentration include the inability to extract insoluble "hyalinised" protein in severe lung injury, and the fact that passage of ions,

proteins and fluid across the alveolar-capillary membrane is a dynamic and rapidly changing process (Folkesson et al., 1991; Hastings et al., 1992; Pittet et al., 1997). With regard to the latter issue Figure 1 illustrated that a relatively low dose of *P. aeruginosa* appeared to cause rapid disruption of epithelial integrity by 4 hours, with a reduction in injury by 24 hours, potentially reflecting early resolution and repair. In contrast, the higher dose of *P. aeruginosa* used in the main body of experiments appeared to induce relatively mild damage at 4 hours, with pronounced damage evident at 24 hours (compare protein concentrations in Table 1 and Figure 2).

Against this background the question of bacterial clearance by control mice is important to the model used here, both for understanding the potential protective effects of human elafin, and for attempting to assess whether bacterial or host mediators are responsible for lung damage.

Importantly there can be no doubt that mice in this study had considerable capacity to clear *P. aeruginosa*, as in control mice the bacterial yield from BALF and lung was still far below the instilled dose, despite the significant lung damage experienced (Figures 5 and 6). The clearance pattern in the model described here is very similar to that reported elsewhere in C57/Bl6 mice administered PAO1 (Cressman et al., 1998) or a PAO1 auxotroph (Yu et al., 1998). It seems likely that a variety of mechanisms are involved in the clearance of *P. aeruginosa* from control mice, with important roles being ascribed to the CXCR2 receptor (Tsai et al., 2000), surfactant protein-A (LeVine et al., 1998), IL-4 (Jain-Voro et al., 1998), and IL-10 (Yu et al., 1998), although the latter mechanism may not be applicable in PAO1 infections (Sawa et al., 1997). Intuitively it seems likely that antimicrobial molecules expressed in the airways contribute to bacterial clearance in mice. Certainly in humans several antimicrobial agents are found in airway secretions under basal conditions (Travis et al., 1999). Interestingly, the ability of mice within each control group to clear bacteria was subject to enormous variation, and indeed variability of biological response was a characteristic of all measured parameters in this chapter.

The model described here was also consistent with published observations regarding the relative kinetics of bacterial eradication and neutrophil influx (Jain-Voro et al., 1998), both being considerably enhanced at 24 hours as compared with 4-6 hours (compare Table 1, Figure 5 and Figure 8). Against this background, the question of whether the major contribution to lung injury in control mice is from bacterial products or host inflammatory cells is difficult to answer. Theoretical

arguments relating to the potential of both *P. aeruginosa* and neutrophils to damage lung tissue during episodes of infection have been well developed (Weiss, 1989; Fick, 1989; Goldberg and Pier, 1996). In the context of this study the increase in BALF protein concentration appeared to be paralleled by increasing neutrophil accumulation at a time when bacterial counts were falling dramatically (compare Table 1, Figure 5 and Figure 8). This may support a role for neutrophils in effecting damage, as may the observation that free MPO was readily detectable in BALF from control mice, suggesting degranulation, and by inference activation (Figure 10). Furthermore in human pneumonia metabolic activity of neutrophils appears to be a post-migratory event (Jones et al., 1997). In contrast, it may be argued that damage (albeit mild) was induced 4 hours after administration of *P. aeruginosa* at a time when bacteria were prevalent and neutrophil accumulation was low (Table 1). Furthermore, the level of neutrophil accumulation at 4 hours (Table 1) was lower than that described in response to LPS in Chapter 4 (Figure 5), when no lung injury was detected. A similar argument has been forwarded to suggest that neutrophils are not important in mediating lung injury upon administration of *P. aeruginosa* in rats (Delclaux et al., 1997).

It is important to acknowledge that the model described here was not designed to distinguish between the relative contributions to injury from bacteria and neutrophils. On balance it seems there is no firm evidence to suggest predominance of either mechanism, but there is a suggestion that bacteria may make a contribution to early lung damage, while neutrophils may contribute to damage in later stages.

A few technical points relating to the model described in this chapter should be mentioned. Firstly, the sudden, massive administration of bacteria to generate lung injury in no way resembles the pathogenesis of pneumonia under "natural circumstances". It is important to emphasise that the primary intention of this study was to examine the effects of Ad-elafin on acute lung injury, rather than to model acquired pneumonia. Irrespective of these considerations, the histological end-point was typical for bronchopneumonia (Figure 4). In this setting it is worth noting that the dose of *P. aeruginosa* used here (approximately 2.2×10^{11} cfu/ml, or approximately 7.5×10^9 cfu per instillation) is higher than that used in most other studies of experimental pneumonia. Why this dose should have been required to achieve reproducible sublethal pneumonia in the mice studied here is unclear.

Secondly, the method of IT administration does involve using a small head of pressure (generated by air in a syringe barrel) to propel instillate, and a minor element of barotrauma in delicate mouse lung cannot be excluded. However this technique was constant for mice in all groups, and it seems unlikely that it contributed to alveolar damage as protein levels in mice receiving no instillate were similar to those receiving PBS alone (see Chapter 4).

Thirdly, the protection conferred by Ad-elafin should potentially be set against the observation that 2 mice died under anaesthesia 5 days after receiving Ad-elafin. However it seems highly unlikely that Ad-elafin is associated with an increased susceptibility to anaesthetic complication, as anaesthetic deaths occur periodically in mice and no other Ad-elafin treated mice died under these circumstances (either in these experiments or in those described in Chapter 4). Furthermore the 2 mice in question appeared healthy before the anaesthetic, and the most likely scenario is that the deaths of these mice was related to an idiosyncratic response to anaesthetic rather than to any effect of elafin.

Fourthly, the absence of *P. aeruginosa* in untreated animals or in virus treated animals not exposed to IT bacteria effectively excluded background colonisation with *P. aeruginosa*.

Finally, it should be emphasised that the relatively non-invasive method of IT delivery in this model (as opposed to the traditional method of surgical exposure of the trachea, followed by cannulation, instillation and closure of the wound) was designed to minimise systemic upset.

6.3.2. The role of Ad-elafin in conferring protection against lung injury mediated by *P. aeruginosa*.

The protection conferred by Ad-elafin against *Pseudomonas* lung injury is best exemplified by the significant reduction in BALF protein (Figure 2). At the time of writing I am not aware of other studies describing a protective effect for molecules with dual anti-elastase/antimicrobial activity against bacterial lung injury *in vivo*. The magnitude of the protective effect is worthy of attention. On one hand it could be argued that the protection was variable, and indeed ineffective in certain mice. On the other hand, bearing in mind the complexity and redundancy inherent in acute inflammation (as described in section 1.2.), it could be argued that a reduction

in severe lung injury of over 40% following a low dose of a single protective vector is extremely encouraging.

The protection was accompanied by the presence of human elafin in murine airways. Two interesting points arise in connection with the elafin concentrations detected in BALF. Firstly, in keeping with data in Chapter 4 (Figure 7), Ad-elafin mice exposed to *P. aeruginosa* for 4 hours had significantly higher elafin levels than did those exposed to PBS. These data again suggest the ability of the mCMV promoter to be activated by inflammatory stimuli. Secondly however, the magnitude of the rise in elafin concentration after application of bacteria was much smaller than that in response to *E. coli* LPS in Chapter 4 (median elafin levels 24 hours after *P. aeruginosa* 8.5 ng/ml as opposed to 120 ng/ml 24 hours after LPS (Figure 7, Chapter 4)). The implication would appear to be either that different inflammatory stimuli have different effects on the mCMV promoter and/or that elafin is less easily detectable (perhaps due to binding or degradation) in more complex inflammatory BALF.

Earlier chapters have demonstrated the ability of elafin to eradicate bacteria and to influence damage associated with both elastase and whole activated neutrophils *in vitro*. As mentioned in section 6.3.1. neither bacteria nor neutrophils emerge as the predominant mediator of injury in the model described in this chapter, and the protection conferred by Ad-elafin is associated with reductions in both bacterial numbers (Figures 5 and 6) and neutrophil-related parameters (Figures 9 and 10). Therefore it is perhaps not surprising that no definitive mechanism emerges to explain protection. Nevertheless, some informative observations can be made.

For example the protection in Ad-elafin treated mice was accompanied by a significant reduction in bacteria retrieved from BALF (Figure 5) and the only parameters to correlate significantly with BALF elafin concentration in this study were the (log transformed) bacterial counts in BALF and lung. This implies (but does not confirm) an important antimicrobial action for elafin in this system. It is notable that the reduction in colonies retrieved from homogenised lung in Ad-elafin mice did not quite attain statistical significance, though there was a trend in this direction. In many respects BALF is the preferred medium for assessing the effect of Ad-elafin on bacterial counts, partly because elafin is a secreted protein, and partly because BALF should reflect the "average" effect from all airways. In contrast, lung colonisation was assessed only in the right lung of each mouse, and it is feasible that

distribution of instillate may favour one lung over another in individual mice, thus introducing variability into the measurement. Importantly, Ad-elafin seemed to be capable of inhibiting haematogenous spread of *Pseudomonas* as reflected in lower rates of bacteria in whole blood or spleen.

The feasibility of elafin as a direct antimicrobial moiety in this system is supported by the data in Chapter 5 and by the ability of cationic antimicrobial peptides to bind *Pseudomonas* LPS *in vitro* (Peterson et al., 1985). Furthermore the human cathelicidin CAP-18 can augment antimicrobial function in a CF bronchial xenograft model (Bals et al., 1999a), and protect mice against the effects of *P. aeruginosa* *in vivo* either when administered as synthetic peptide (Kirikae et al., 1998) or via an adenoviral vector (Bals et al., 1999b).

If the effect of Ad-elafin in this model is indeed to eradicate bacteria independently of the effect of neutrophils, it must be re-emphasised that the effect is not protective in all mice, and that the effect must be additive to that of powerful endogenous clearance mechanisms. Furthermore, although the numbers of mice studied in Table 1 are small, it would appear that elafin exerts its principal effect after 4 hours, at which point bacterial loads are already falling. The inference may be that elafin has an indirect antimicrobial effect through induction of as yet unidentified mechanisms.

Elafin's capacity to inhibit elastase clearly raises the possibility of an anti-elastase effect in mediating protection in the model described here. Certainly elastase is a feasible mediator of lung injury both in pneumonia generally, and in the setting of airway disease associated with *Pseudomonas*. For example free, active elastase can be retrieved in BALF from humans with pneumonia (Boutten et al., 1996), and elastase activity in circulating granulocytes is increased during experimental canine pneumonia (Lonky et al., 1980). In the context of *P. aeruginosa* associated with CF, HNE appears to be able to reduce opsonisation and phagocytosis by cleaving complement receptors on neutrophils (Berger et al., 1989; Tosi et al., 1990), while evidence suggests that HNE (as opposed to *Pseudomonas* proteases) is a key mediator of lung injury (Meyer and Zimmerman, 1993; Venaille et al., 1998). In specific relation to experimental pneumonia associated with PAO1, free elastase activity is generated in hamster airways after IT administration (Melby et al., 1985). Finally, *Pseudomonas* elastase is capable of inactivating human α_1 -PI and SLPI

(Sponer et al., 1991) and it remains feasible that anti-elastases are functionally depleted in the presence of *P. aeruginosa*.

Despite this background however, no elastase activity was detected in BALF from any of the mice studied in this chapter. Indeed, the EIA was lower in Ad-elafin mice than in controls (Figure 11), and human elafin was calculated to comprise less than 1% of the EIA, if indeed it contributed at all. It must therefore be concluded that the effect of Ad-elafin in this study is not attributable to inhibition of free elastase in the airways. Attention should therefore be turned to the number and function of neutrophils if an anti-neutrophil activity of Ad-elafin is to be invoked. Before doing so however, it is worth briefly considering the EIA in these experiments. Both the reduction in EIA in Ad-elafin mice (Figure 11) and the strong correlation between EIA and protein concentration in BALF (Figure 12) suggest that EIA is generated as a response to injury. A similar effect has been described in humans with acute lung disease (Sallenave et al., 1999b). The exact composition of the EIA remains unexplained, and the tools to dissect this question were not immediately available at the time these experiments were performed. With regard to the two major antiproteinases in murine serum, α_1 -PI is thought not to be a significant acute phase reactant (Baumann et al., 1983), and contrapsin does not inhibit elastase (Takahara and Sinohara, 1983). Interestingly, murine SLPI mRNA levels are significantly increased in the lungs of C57/Bl6 mice exposed to Streptococcal pneumonia (Abe et al., 1997) and murine SLPI may potentially have contributed to EIA in this study.

Whether Ad-elafin's protective effect in this model had anything to do with neutrophil migration is debatable. The absolute number of neutrophils in BALF from Ad-elafin mice was in fact higher than in Ad-lacZ mice, despite injury being significantly less in the former group (compare Figures 2 and 8). However there was a trend towards fewer neutrophils in lung tissue among Ad-elafin mice (Figure 9). At this juncture it is worth reflecting that Ad-elafin administration was associated with a significant increase in neutrophil recruitment in Chapter 4 (Figure 5). In interpreting this apparent discrepancy it should be observed that BALF neutrophil counts in PBS/*Pseudomonas* mice in this chapter are 13 times higher than those in PBS/LPS mice described in Chapter 4 (Figure 5), reflecting the much more potent inflammatory stimulus in this chapter. Furthermore, elafin levels were 14 times higher in Ad-elafin/LPS mice (Figure 7, Chapter 4) than in Ad-elafin/*Pseudomonas* mice in this chapter. It remains feasible that elafin may only facilitate neutrophil

recruitment when in high concentration and/or in the presence of intact lung parenchyma.

On balance the small, statistically non-significant difference between neutrophil counts in Ad-lacZ and Ad-elafin mice (Figures 8 and 9), and the striking difference in BALF protein levels when comparing the same groups mitigates against a direct role for neutrophil migration in explaining the protective effect of Ad-elafin.

In considering whether Ad-elafin's protective effect is via an effect on neutrophils, it is finally necessary to consider neutrophil activation. Figure 10 suggests that Ad-elafin administration was associated with markedly reduced degranulation of airway neutrophils in some mice, though the variability in individual mice dictated that the effect was not statistically significant. This raises the intriguing possibility that elafin may inhibit degranulation of neutrophils, which could theoretically explain the tissue protection conferred in the experiments described in this chapter, and the lack of tissue damage despite a doubling in airway neutrophils in Chapter 4, Figure 5. It must be emphasised that there is no firm evidence to support this hypothesis, and it should therefore be regarded simply as an area requiring further attention.

Turning briefly to the role of macrophages, it is unlikely that these cells induced tissue injury associated with *P. aeruginosa*, as numbers were higher in Ad-elafin mice than in controls. It is worth recalling that alveolar macrophages were also increased among Ad-elafin/LPS mice in Chapter 4, and an effect of elafin on macrophage recruitment cannot be excluded at this point.

In attempting to summarise this section it is clear that Ad-elafin augmented the defence of murine airways against *P. aeruginosa*. While the mechanism involved is not clear, the available evidence suggests that it is at least in part mediated by acceleration in the rate of bacterial clearance, whether this effect be direct or indirect.

6.3.3. The potential protective effect of Ad-lacZ.

At various points in this chapter a trend has arisen towards a protective effect against *Pseudomonas* in Ad-lacZ treated mice as compared with PBS treated mice.

This was particularly striking when considering bacterial load in BALF (Figure 5), but was also apparent when considering albumin concentration and neutrophil count in BALF (Figures 3 and 8), and the number of *Pseudomonas* colonies retrieved from blood and spleen. Furthermore, BALF protein concentration was lower in Ad-lacZ treated mice than in PBS treated mice studied 4 hours after administration of *Pseudomonas* (Table 1). This trend is to some degree mirrored by relative reductions in HNE-mediated cell detachment (Chapter 3, Figure 8), LPS-mediated neutrophilia (Chapter 4, Figure 5), and IL-8 levels released from A549 cells (Chapter 4, Figure 11). It must be strongly emphasised that none of these differences reached statistical significance (indeed no significant differences were found comparing vehicle treatment with Ad-lacZ treatment *in vitro* or *in vivo* in any of the experiments described in this thesis). Furthermore, a pro-inflammatory tendency was observed for Ad-lacZ when studying neutrophil migration *in vitro* (Chapter 4, Figure 10).

Nevertheless the apparent trend towards a protective effect of Ad-lacZ *in vivo* is intriguing and to some extent is consistent with findings elsewhere. For example replication deficient adenovirus has been shown to stimulate clearance of *Helicobacter felis* from the stomach of C57/Bl6 mice in an IL-12- and IFN- γ -dependent manner (Jiang et al., 1999), and a similar null adenovirus appeared to enhance clearance of *P. aeruginosa* PAO1 from the lungs of rats (Worgall et al., 1999b). The implication may be that low grade stimulation of innate or adaptive immunity by adenovirus may promote bacterial clearance under specific conditions. This may suggest potential therapeutic applications, and in this context it is worth noting that reduction in tumour size has been linked to the immunogenicity of adenoviral vectors in animal models (Song et al., 1997b).

6.3.4. Gene therapy for pulmonary infection.

The finding that Ad-elafin confers protection against acute inflammatory lung injury mediated by *P. aeruginosa* adds to emerging data describing a role for gene therapy in infection.

To a large extent this field evolved with the concept that key cytokines play important roles in the regulation of inflammation. For example adenovirus encoding IL-12 was shown to protect mice against the lethal effects of *Klebsiella pneumoniae* (Greenberger et al., 1996). At around the same time it was demonstrated that IL-10 gene therapy could dampen the expression of inflammatory cytokines in murine

endotoxaemia (Xing et al., 1997), with subsequent demonstration that adenovirus encoding IL-10 could protect against tissue injury in a porcine model of pneumonia induced by *Actinobacillus pleuropneumoniae* (Morrison et al., 2000).

Extending this theme further, IT adenoviral transfer of interferon- γ has been shown to enhance clearance of *P. aeruginosa* from the lungs of rats (Lei et al., 1997). This intervention was also demonstrated to enhance eradication of *Pneumocystis carinii* in an elegant model of pulmonary infection in CD4 depleted mice (Kolls et al., 1999).

These intriguing strategies have been accompanied recently by novel variants all concerned with clearance of *Pseudomonas*. For example it was demonstrated that pulmonary host defences are impaired during systemic sepsis partly on account of local TNF depletion, and that IT TNF gene therapy restored clearance of *P. aeruginosa* from the lung in the course of experimental sepsis (Chen et al., 2000). In addition, a strategy aimed at directly enhancing antimicrobial activity in the lung showed that adenovirus encoding the endogenous antibiotic CAP-18 enhances clearance of *P. aeruginosa* (Bals et al., 1999b). Finally, a fascinating strategy using adenovirus encoding Fc γ receptors conferred the capacity for phagocytosis on pulmonary epithelial cells, thus enhancing clearance of *P. aeruginosa* (Worgall et al., 1999b).

While the work presented in this chapter and the studies described in this section suggest novel therapeutic strategies for acute bacterial lung injury, it should be noted that all of the interventions used were preventive in that gene therapy was administered prior to bacterial pathogens. A major challenge will be to develop gene therapy strategies which promote resolution of pre-existing pulmonary inflammation. It is already known that such inflammation impairs the transfer of adenovirus (Otake et al., 1998; van Heeckeren et al., 1998), and imaginative strategies will be required to define and overcome the barriers to effective transfection.

In summary, the work in this chapter has demonstrated that elafin gene augmentation is capable of reducing tissue injury in an animal model of acute inflammation mediated by *P. aeruginosa*, an organism associated with intrinsic antimicrobial resistance. This effect was achieved using a single IT administration of adenovirus at a dose previously shown to produce minimal vector-induced inflammation. Although the mechanism of protection remains uncertain, the

available evidence suggests that enhanced clearance of bacteria was at least partly responsible.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The experiments described in this thesis have confirmed that elafin gene augmentation is feasible both *in vitro* and *in vivo*. They have shown that elafin augmentation protects pulmonary epithelium against damage mediated by HNE and by activated human neutrophils. This led to experiments demonstrating that elafin gene augmentation may enhance LPS-mediated pulmonary neutrophilia in mice. The characteristics of elafin suggested intrinsic antimicrobial properties, and these were confirmed by demonstrating activity against Gram-positive and Gram-negative pulmonary pathogens.

These data prompted experiments in which elafin gene augmentation was found to confer murine lungs with significant protection against sublethal pneumonia induced by *P. aeruginosa*. Collectively these findings infer an important role for elafin in host innate immunity and suggest that elafin gene augmentation may have therapeutic potential.

An additional finding was that elafin expression could be achieved using doses of adenovirus low enough to circumvent overt vector-induced inflammation. Furthermore it was found that the mCMV promoter within the Ad-elafin vector was markedly up-regulated in response to inflammatory stimuli, as reflected by a 20-fold increase in elafin expression in response to LPS *in vivo*. Together these findings suggest means of optimising expression in adenoviral gene therapy protocols whilst minimising viral toxicity.

While these experiments may have furthered understanding of elafin biology and of the potential application of antiproteases in gene therapy protocols, a huge number of important questions remain unanswered and should form the focus of future research.

For example the interaction of elafin with other molecules central to innate immunity requires to be elucidated, including the relationship with other anti-elastases in clearing HNE from the airways and interstitium. The role (if any) for interstitial binding of elafin in inflammatory responses requires to be characterised in the lung. The effect of oxidants on the methionine in the active site of elafin requires attention, as does the question of whether elafin fragments found in human airway secretions retain biological activity. The effect of elafin on neutrophil and alveolar

macrophage function requires definition, and the conditions under which elafin helps recruit neutrophils need to be clarified. Importantly the mechanism for elafin's antimicrobial activity also requires to be unravelled.

Finally, this work set out with the ultimate aim of determining whether elafin gene augmentation could protect tissue against inflammatory injury. While the principle of protection has been demonstrated by delivering Ad-elafin prior to application of injurious stimuli, it remains to be seen whether (and under which conditions) elafin gene augmentation can ameliorate existing inflammation. Dissecting these questions may allow further progress towards the goal of defining conditions for safe, effective, and sustained gene therapy.

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PUBLICATIONS ARISING FROM THIS WORK

Three publications have arisen directly from the work presented in this thesis. Copies are found in the following section.

In addition I contributed to one other original paper during my time in the lab (Sallenave et al., 1998), and to a review article on respiratory gene therapy (Simpson et al., 2000). Copies of these articles are not included in the following section, but both are cited in the body of the thesis.

Adenoviral Augmentation of Elafin Protects the Lung Against Acute Injury Mediated by Activated Neutrophils and Bacterial Infection¹

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During acute pulmonary infection, tissue injury may be secondary to the effects of bacterial products or to the effects of the host inflammatory response. An attractive strategy for tissue protection in this setting would combine antimicrobial activity with inhibition of human neutrophil elastase (HNE), a key effector of neutrophil-mediated tissue injury. We postulated that genetic augmentation of elafin (an endogenous inhibitor of HNE with intrinsic antimicrobial activity) could protect the lung against acute inflammatory injury without detriment to host defense. A replication-deficient adenovirus encoding elafin cDNA significantly protected A549 cells against the injurious effects of both HNE and whole activated human neutrophils in vitro. Intratracheal replication-deficient adenovirus encoding elafin cDNA significantly protected murine lungs against injury mediated by *Pseudomonas aeruginosa* in vivo. Genetic augmentation of elafin therefore has the capacity to protect the lung against the injurious effects of both bacterial pathogens resistant to conventional antibiotics and activated neutrophils. *The Journal of Immunology*, 2001, 167: 1778–1786.

Toxic products released by neutrophils, especially human neutrophil elastase (HNE),³ have been implicated in the pathogenesis of a variety of inflammatory disorders characterized by pulmonary neutrophilia, including cystic fibrosis (CF), non-CF bronchiectasis, emphysema, and bacterial pneumonia (1–4). The therapeutic rationale for depleting circulating neutrophils in such conditions is negated by the propensity for overwhelming sepsis in neutropenic patients and by the observation that mice deficient in neutrophil elastase are predisposed to Gram-negative sepsis (5). This argument applies especially in the context of bacterial pneumonia. In such situations, inhibition of extracellular HNE by agents also possessing antimicrobial properties would be an attractive strategy in attempting to protect the lung from inflammatory injury.

Inhibitors of HNE are thought to comprise part of the human innate immune system. Three distinct antielastases have been described in the human lung: α_1 -protease inhibitor, secretory leukocyte protease inhibitor (SLPI), and elafin (elastase-specific inhib-

itor) (6). Elafin (7, 8) is a potent inhibitor of HNE and proteinase 3 produced in the skin (9–11), and in the airways (12), which is up-regulated in response to early inflammatory cytokines such as TNF and IL-1 (13). Elafin, along with SLPI, also shares characteristics with antimicrobial defensin-like molecules in being a low m.w. cationic peptide with the ability to eliminate pulmonary pathogens (14–16).

We therefore hypothesized that local augmentation of elafin by constitutive lung cells would confer protection against inflammatory injury, especially when the lung was challenged by bacterial pathogens. In these studies, we used an adenoviral gene transfer approach for a number of reasons, including the natural tropism of adenovirus for the respiratory epithelium, the potential to regulate transgene expression using carefully selected promoters (17, 18), and the well-described ability to express antielastases in the lung using adenoviral gene therapy (17, 19). Against this background, we demonstrate for the first time that A549 cells transfected with adenovirus encoding human elafin in vitro protects not only against HNE-induced damage but also against injury caused by primed and activated human neutrophils. We also show for the first time that intratracheal (i.t.) transfer of adenovirus encoding a human gene with dual antielastase and antimicrobial properties protects murine lungs against acute inflammatory injury caused by *Pseudomonas aeruginosa*, a bacterial pathogen commonly resistant to conventional antibiotics (20, 21). Our findings extend the observed potential for gene therapy strategies in the management of pulmonary injury and infection (22–26) by demonstrating that the protection conferred in vivo was achieved using doses of adenovirus which were not themselves associated with significant vector-induced airway inflammation.

Materials and Methods

Adenoviral constructs

The adenoviral constructs used have been described in detail elsewhere (17, 18). In brief, human elafin cDNA (encoding full length elafin) was cloned into pDK6 downstream of a 1.4-kb fragment of the murine CMV promoter (27). PDK6 and pBHG10 were used to cotransfect 293 cells

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³ Abbreviations used in this paper: HNE, human neutrophil elastase; Ad-elafin, E1-, partially E3-deleted adenovirus encoding human elafin cDNA; Ad-lacZ, E1-, partially E3-deleted adenovirus encoding lacZ cDNA; BALF, bronchoalveolar lavage fluid; CF, cystic fibrosis; ELA, elastase-inhibitory activity; i.t., intratracheal; mKC, murine keratinocyte-derived chemokine; moi, multiplicity of infection; MPO, myeloperoxidase; PAF, platelet-activating factor; SLPI, secretory leukocyte protease inhibitor.

(which generate the product of the adenoviral E1 region in *trans*). Homologous recombination resulted in the generation of E1-, partially E3-deleted adenovirus encoding human elafin cDNA (Ad-elafin). The second virus used (E1-, partially E3-deleted adenovirus encoding *lacZ* cDNA (Ad-*lacZ*)) was constructed in the same manner with the exception that *lacZ* cDNA (under the control of the same murine CMV promoter fragment) was cloned in place of elafin cDNA (17, 18).

Preparation of human neutrophils

Fresh citrated whole blood was obtained from healthy volunteers. Neutrophils were prepared by dextran sedimentation and Percoll gradient extraction using a standard technique described elsewhere (28). BSA (Sigma, Poole, U.K.) at a final concentration of 1% was added to cell supernatants before addition of neutrophils. Neutrophils were activated by the sequential addition to cell supernatants of platelet-activating factor (PAF) (Calbiochem, Nottingham, U.K.) at a final concentration of 10^{-9} M, and fMLP (Calbiochem), final concentration 10^{-7} M.

In vitro transfection experiments

A549 cells were used in in vitro transfection experiments. A549 cells are derived from bronchioloalveolar cell carcinoma (29) and share several phenotypic characteristics with type II alveolar epithelial cells (29, 30). Indeed, several features of A549 cells have been reproduced in primary cell lines; in particular elafin is expressed by pulmonary epithelial cell lines and up-regulated in response to IL-1 and TNF, features characteristic of A549 cells (13, 30).

A549 cells were incubated at 37°C (and 5% CO₂) in DMEM (Sigma) containing 10% FCS (Sigma), penicillin G (final concentration, 100 U/ml; Life Technologies, Paisley, U.K.), streptomycin sulfate (final concentration, 100 µg/ml; Life Technologies), and 1% L-glutamine (final concentration, 2 mM; Life Technologies) and grown to confluence in 24-well plates (Corning Costar, High Wycombe, U.K.). Cells were washed with PBS (Sigma) and then incubated for 30 min at 37°C with one of three treatments: Ad-elafin in DMEM containing 5% FCS; Ad-*lacZ* in DMEM containing 5% FCS; or DMEM containing 5% FCS with no virus added. Adenovirus was applied at a multiplicity of infection (moi) of 50 PFU (except in dose-response experiments where adenovirus was applied over the dose range 0–50 PFU). Cells were washed with PBS, and the medium was changed to DMEM containing 10% FCS, before incubation overnight at 37°C. Cells were washed extensively using PBS and incubated at 37°C in serum-free DMEM before the addition of either purified HNE at a final concentration of 4 µg/ml (Elastin Products, Owensville, MO) or 2×10^6 human neutrophils (activated by PAF and fMLP as above). After 16 h, the damage to the monolayer was assessed morphologically by light microscopy or by counting the number of A549 cells liberated into the supernatant.

In a variation of these experiments, A549 cells were radiolabeled with ¹¹¹In, using a variation on methods described previously (31). ¹¹¹In (DuPont, NEN Life Sciences, Brussels, Belgium) was incubated with 4×10^{-3} M tropolone (Sigma) for 1 min. ¹¹¹In (3 µCi) was added to A549 cells at the end of the overnight incubation in DMEM containing 10% FCS (final concentration of tropolone, 4×10^{-4} M). Damage to the monolayer after addition of human neutrophils was assessed 16 h later by measuring radioactivity in the supernatant using a scintillation analyzer (model 1900TR; Canberra Packard, Pangbourne, U.K.). It is well established that neutrophils from different healthy individuals may respond very differently to the effects of priming agents. In preliminary experiments, 2×10^6 neutrophils, activated with 10^{-9} M PAF and 10^{-7} M fMLP, caused 30–70% damage to untransfected A549 cells at 16 h in the significant majority of individuals. A protocol was therefore devised whereby neutrophils causing <30% or >70% damage to untransfected monolayers were excluded from analysis. In subsequent experiments, 10 healthy volunteers donated neutrophils. In eight of these the protocol was satisfied (in one of the remaining cases, 100% of untransfected A549 cells were damaged at 16 h, and in the other no untransfected A549 cells were morphologically damaged at 16 h).

To correct for the theoretical risk of nonspecific leakage of ¹¹¹In from A549 cells, radioactivity in supernatants from cells that did not have neutrophils applied was subtracted from the count in neutrophil-treated cells for all conditions studied.

Preparation of *P. aeruginosa* PAO1

P. aeruginosa PAO1, a well-characterized type strain used in many genetic (21) and animal studies, and the first strain of *P. aeruginosa* to be fully sequenced (32), was inoculated into nutrient broth (Oxoid, Basingstoke, U.K.) containing 0.5% yeast extract (Difco, Detroit, MI) and incubated overnight at 37°C in an orbital incubator at 200 rpm (3). The culture was centrifuged at 4500 rpm at room temperature for 15 min, and the bacteria

were washed in 0.01 M phosphate buffer, pH 7.0. The bacterial suspension used in in vivo experiments was prepared in the same buffer to provide a population density of $\sim 2.2 \times 10^{11}$ CFU/ml when cultured on *Pseudomonas* Isolation Agar (Difco).

In vivo experiments

Female C57BL/6 mice between 6 and 8 wk old were from Harlan Olac (Bicester, U.K.). Mice were anesthetized using i.p. avertin (~ 10 µl/g body weight; avertin comprised 1.25% 2,2,2-tribromoethanol (Aldrich, Gillingham, U.K.) and 2.5% 2-methyl-2-butanol (Sigma)). In all experiments involving i.t. administration, the vocal cords were viewed directly, and a blunt catheter was passed beyond them according to methods previously described by our group (33). Treatments of known volume were instilled directly, and body fluids or organs extracted as described below. Preliminary experiments in which trypan blue was administered i.t. consistently revealed a similar distribution of dye to all lobes of the lung macroscopically (data not shown). All mice were euthanized under anesthesia (using avertin) by transection of the abdominal aorta.

In one set of preliminary experiments, four mice that had not received any form of i.t. treatment were anesthetized as above and euthanized. The lungs and trachea were removed en bloc and bronchoalveolar lavage fluid (BALF) was obtained by instillation of two separate aliquots of 250 µl sterile PBS. BALF was serially diluted, inoculated onto *Pseudomonas* Isolation Agar, and incubated at 37°C overnight. The remaining BALF was centrifuged at 2000 rpm for 10 min at 4°C. The pellet was resuspended in PBS; the total cell count was established and cytopins were prepared to estimate differential cell count. The supernatant was stored at -40°C before further use.

In a further set of preliminary experiments mice were anesthetized as above and received an i.t. instillation of either Ad-elafin (3×10^7 PFU) suspended in PBS, Ad-*lacZ* (3×10^7 PFU) suspended in PBS, or PBS alone ($n = 4$ in each group). Five days later, mice were anesthetized in the same way and given i.t. PBS. After 24 h, mice were euthanized as above. BALF was prepared to establish *P. aeruginosa* PAO1 colony counts and differential cell counts as described above (with the exception that BALF was prepared using aliquots of 250 µl and then 200 µl).⁴

In the ensuing experiments, Ad-elafin (3×10^7 PFU) suspended in PBS, Ad-*lacZ* (3×10^7 PFU) suspended in PBS, or PBS alone were instilled by i.t. injection in a final volume of 40 µl (PBS group $n = 18$, Ad-*lacZ* group $n = 20$, Ad-elafin group $n = 16$). Five days later, mice were anesthetized in the same way, and a direct i.t. instillation of *P. aeruginosa* PAO1 was administered ($\sim 2.2 \times 10^{11}$ CFU/ml suspended in 0.01 M phosphate buffer; final volume, 34 µl). In preliminary experiments, we found that *P. aeruginosa* PAO1 at this predetermined dose consistently produced a sublethal pneumonia. Twenty-four hours after administration of *P. aeruginosa* PAO1, mice were anesthetized and euthanized by transection of the abdominal aorta. Whole blood was retrieved; an aliquot of 100 µl was inoculated onto *Pseudomonas* Isolation Agar and incubated at 37°C overnight. The spleen was stored in 0.01 M phosphate buffer and homogenized for 30 s using an Omni hand-held homogenizer (Camlab, Cambridge, U.K.); an aliquot of 100 µl was inoculated onto *Pseudomonas* Isolation Agar and incubated at 37°C overnight. The lungs and trachea were removed en bloc, and BALF was obtained as described above. *P. aeruginosa* colony counts and differential cell counts in BALF were established as described above. The left lung was weighed, snap frozen in liquid nitrogen, and stored at -80°C . Immediately upon thawing, the lung was placed in homogenization buffer (100 mM sodium acetate, 20 mM EDTA, 1% hexadecyltrimethylammonium bromide (all Sigma)) as described elsewhere (34), and homogenized for 1 min. The homogenate was centrifuged at 10,000 rpm for 30 min at room temperature. The supernatant was used immediately to determine the concentration of myeloperoxidase (MPO) in the homogenized lung (as an index of the number of neutrophils in both pulmonary parenchyma and circulation) (34).

In separate experiments, three additional mice from each group (i.e., PBS then *P. aeruginosa* PAO1; Ad-*lacZ* then *P. aeruginosa* PAO1; Ad-elafin then *P. aeruginosa* PAO1) were euthanized 24 h after administration of *P. aeruginosa* PAO1, and the lungs were removed en bloc before fixing in formalin and embedding in paraffin wax. Sections 3 µm thick were cut and stained using hematoxylin and eosin.

Assays

Levels of human elafin Ag were measured using a sandwich ELISA available in-house, performed on 96-well plates (Linbro; Flow Laboratories).

⁴ Other data relating to these control mice have been submitted elsewhere, but the data presented in this article do not appear elsewhere.

McLean, VA). In brief, the primary Ab was polyclonal rabbit anti-human elafin Ig, gelatin (BDH, Poole, U.K.) was used as a blocking agent, the sample (or purified recombinant elafin as standard) was applied, and the secondary Ab was biotinylated polyclonal rabbit anti-human elafin Ig (biotin was from Pierce, Rockford, IL). Streptavidin-HPO complex (Sigma) and then chromogenic substrate (2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); Sigma) were added sequentially and the OD₅₅₀ of the product was quantified using a Dynatech M5000 Plate Reader (Dynex, Billingham, U.K.).

Elastase activity was measured by applying the elastase-specific chromogenic substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (Sigma), and measuring change of OD₄₀₅. Elastase-inhibitory activity (EIA) was measured by adding sample (serially diluted in buffer (50 mM Tris, 0.1% Triton, 0.5 M sodium chloride, pH 8); final volume, 10 μ l), or buffer alone to a known quantity of purified HNE (50 ng in 10 μ l) and incubating for 30 min at 37°C before adding 50 μ l *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide and measuring change of OD₄₀₅. Plots of OD (reflecting HNE activity) against concentration of sample were constructed. As described elsewhere (35), EIA was derived from extrapolation of the curve to the abscissa.

MPO activity was calculated by addition of chromogenic substrate (0.1 mg/ml tetramethylbenzidine (DAKO, Denmark), 0.03% hydrogen peroxide (Sigma) in 0.1 M sodium acetate, pH 4.9) and measurement of the change of OD₆₃₀. To measure MPO activity in homogenized lung supernatants, samples were diluted in homogenization buffer, and 100- μ l aliquots (in triplicate) added to 100 μ l chromogenic substrate. To measure MPO activity in BALF, 50- μ l aliquots of BALF were treated with 10 μ l sodium acetate buffer, pH 4.2, before addition of 100 μ l chromogenic substrate.

Protein was measured using the bicinchoninic acid method (Pierce) using purified albumin (Pierce) as standard.

Commercial ELISA kits were used to measure concentrations of murine albumin (Bethyl Laboratories, Montgomery, TX) and human IL-8 (R&D Systems, Abingdon, U.K.).

Measurements of murine keratinocyte-derived chemokine (mKC) concentration in BALF, using an ELISA, were kindly performed by Professor T. Standiford (University of Michigan, Ann Arbor, MI).

Statistics

The parameters studied were not normally distributed, and nonparametric tests were applied. For comparisons involving three groups, the Kruskal-Wallis test was used, and comparisons between pairs of groups were performed using the Mann-Whitney *U* test. Paired data were studied using Wilcoxon's rank sum test. Nominal data were compared using the χ^2 test. Correlations were studied using Spearman's rank correlation test.

Results

Effects of adenoviral augmentation of elafin on damage mediated by HNE and activated human neutrophils in vitro

Transfection of A549 cells with Ad-elafin resulted in a dose-dependent increase in both secretion of elafin Ag and antielastase activity, indicating that elafin production was efficient and that the elafin produced was functionally active (Fig. 1). Adenoviral transfection did not increase secretion of IL-8 above that from untransfected cells. Indeed Ad-lacZ and Ad-elafin transfection (each at an moi of 50 PFU) resulted in IL-8 levels that were, respectively, 54 and 63% of those from untransfected cells ($n = 3$; $p = 0.05$ comparing the three groups).

Ad-elafin significantly protected A549 cells against injury induced by HNE as assessed morphologically (Fig. 2, a-c). This observation was supported by a significant reduction in the number of cells released from pulmonary epithelial monolayers after treatment with HNE (Fig. 2d). The protection conferred was associated with significant and almost complete inhibition of HNE (Fig. 2e).

When human neutrophils, activated with a combination of priming agents (PAF) and secretagogues (fMLP), were used in place of purified HNE, morphological evidence of protection was again conferred by Ad-elafin (Fig. 3, a and b). This was associated with a reduction in release of ¹¹¹In from radiolabeled epithelial cells (Fig. 3c). The protection conferred by Ad-elafin was associated with a significant reduction in measured HNE activity (Fig. 3d).

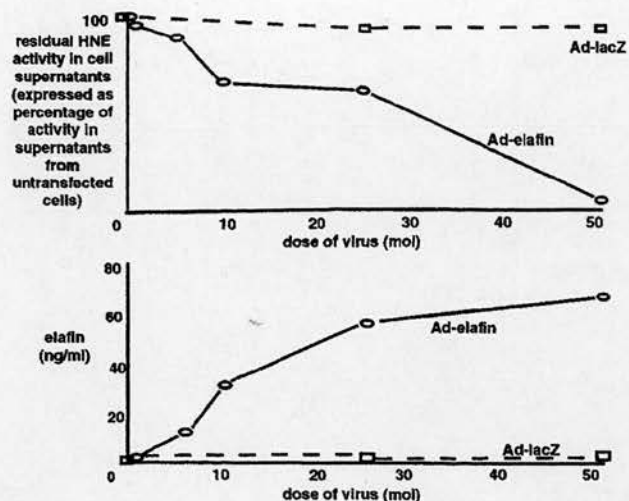


FIGURE 1. Transfection of pulmonary epithelial cells with Ad-elafin results in dose-dependent production of elafin and anti-elastase activity. A549 cells were treated with Ad-elafin (moi 0–50 PFU) or Ad-lacZ (moi 0–50 PFU) for 30 min at 37°C. Cells were washed with PBS to remove residual virus and then incubated overnight at 37°C in medium containing serum. Cells were extensively washed with PBS to remove serum and incubated at 37°C for 24 h in serum-free medium. Supernatants were retrieved 16 h later. Two aliquots of supernatant were prepared. A known quantity of HNE was added to the first aliquot, and residual HNE activity measured using a chromogenic substrate assay specific for HNE. In the second aliquot, elafin concentration was determined by ELISA. *Bottom*, Dose-dependent increase in elafin secretion after Ad-elafin transfection (heavy line) but not after Ad-lacZ transfection (broken line); *top*, corresponding dose-dependent generation of antielastase activity after Ad-elafin (heavy line), but not Ad-lacZ (broken line) transfection.

Preliminary in vivo experiments

In mice receiving no i.t. treatments (i.e., normal mice), the median total cell count in BALF was 1.84×10^5 , with macrophages comprising >95% of the count in all animals. The remaining cells in BALF were almost exclusively neutrophils, as is characteristic of BALF from normal mice. Median protein concentration in BALF was 0.45 g/L (range, 0.43–0.53 g/L). In mice receiving i.t. vector and/or PBS, very similar trends were observed (Table I). Total cell counts were similar, and the alveolar macrophage was the predominant cell type, with the neutrophil comprising a median of 0.7% of cells in BALF among PBS/PBS mice, 3.4% in Ad-lacZ/PBS mice, and 0.6% of Ad-elafin/PBS mice (no significant difference). Similarly, adenovirus administration itself did not significantly influence the concentration of protein in BALF characteristic of untreated animals or those treated sequentially with vehicle alone (Table I). Therefore, adenovirus administration per se was not associated with significant airway inflammation as assessed by cellular content and protein concentration of BALF. No bacterial growth was obtained on *Pseudomonas* Isolation Agar plates inoculated with BALF (Table I), blood, homogenized spleen, or homogenized lung from untreated, vehicle-treated, or virus-treated mice, reflecting the absence of either commensal or contaminating *P. aeruginosa* in our laboratory.

Effects of Ad-elafin transfection on lung injury mediated by *P. aeruginosa* in vivo

Delivery of Ad-elafin i.t. followed by *P. aeruginosa* PAO1 resulted in a median human elafin concentration in BALF of 8.5 ng/ml (interquartile range, 5.9–10.4 ng/ml). No human elafin was detected in any of the mice given Ad-lacZ (as viral control) or PBS

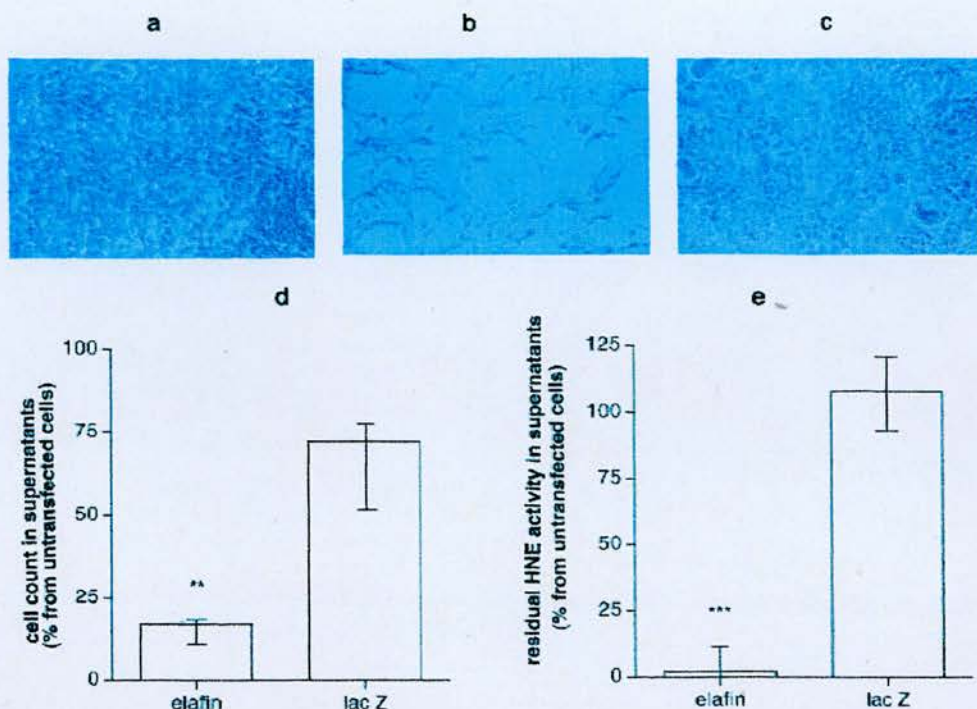


FIGURE 2. Ad-elafin transfection protects pulmonary epithelial cells against HNE in vitro. A549 cells were treated with Ad-elafin (moi 50 PFU), Ad-lacZ (moi 50 PFU), or medium alone (untransfected cells) for 30 min at 37°C. Cells were washed with PBS to remove residual virus and then incubated overnight at 37°C in medium containing serum. Cells were extensively washed with PBS to remove serum and incubated at 37°C for 48 h in serum-free medium. HNE was then added directly to the supernatant (final concentration, 4 μ g/ml) and incubated at 37°C for 16 h. Photomicrographs of cell layers were taken (using a blue filter to optimize contrast), cells in the supernatant were counted, and the residual HNE activity of supernatants was measured using a chromogenic substrate assay specific for HNE. *a*, Untransfected cells, with no HNE added; *b*, Ad-lacZ-treated cells, after addition of HNE (appearances of untransfected cells treated with HNE were almost identical); *c*, Ad-elafin-treated cells, after addition of HNE; *d*, cell counts in supernatants retrieved after addition of HNE ($n = 6$). Results were expressed relative to the count in supernatants from untransfected cells, which was regarded as 100% in each experiment. Values represent medians (and interquartile ranges) from the six experiments performed. **, Significantly lower compared with either of the other treatments, $p < 0.01$. *e*, Residual HNE activity in supernatants ($n = 6$). Results were expressed relative to the HNE activity in supernatants from untransfected cells, which was regarded as 100% in each experiment. The values shown represent medians (and interquartile ranges) from the six experiments performed. ***, Significantly lower compared with each of the other treatments, $p < 0.005$.

(as nonviral control) and then *P. aeruginosa* PAO1, confirming that antielafin IgG does not cross-react with murine or bacterial proteins. Histologically, the administration of *P. aeruginosa* PAO1 in control mice was associated with a patchy, multilobar pneumonia, associated with extravasation of neutrophils into alveolar airspaces (Fig. 4, *a* and *c*) with associated protein leak. These effects were much less pronounced in mice receiving Ad-elafin (Fig. 4*b*) in which there was a significant reduction in the concentrations of protein and albumin in BALF (Fig. 4*d*).

The protection conferred by Ad-elafin was associated with a significant reduction in the quantity of bacteria retrieved from BALF (Fig. 4*d*). Indeed, among the mice receiving Ad-elafin, the concentration of elafin in BALF significantly and inversely correlated with the number of *P. aeruginosa* PAO1 colonies (log transformed) in BALF ($r = -0.75$; $p = 0.02$). Administration of Ad-elafin was also associated with a significantly lower incidence of bacterial growth in blood (colonies detected in 31% of Ad-elafin mice, 44% of Ad-lacZ mice, and 61% of PBS mice, $p < 0.05$) and spleen (colonies detected in 19% of Ad-elafin mice, 40% of Ad-lacZ mice, and 44% of PBS mice, $p < 0.05$).

In none of the mice studied was elastase activity detected in BALF, reflecting a relative excess of elastase inhibitors in BALF from all animals. However, the total EIA in BALF was lower in the Ad-elafin group than in either of the control groups (Fig. 5).

In addition, BALF from mice receiving Ad-elafin contained significantly lower concentrations of the murine neutrophil chemo-

kine mKC than did BALF from mice receiving control virus (Table II). In keeping with this finding, a trend was observed toward lower concentrations of MPO in homogenized lung (reflecting lung neutrophil count) in mice receiving Ad-elafin (Table II). Indeed, considering all mice together, there was a significant correlation between the concentration of mKC in BALF and the concentration of MPO in homogenized lung ($r = 0.43$, $p < 0.005$). A trend toward lower levels of free MPO in BALF (reflecting neutrophil degranulation products) was also found in mice receiving Ad-elafin (Table II).

Discussion

We believe these data represent the first description of protection against acute lung injury conferred by genetic augmentation of a human pulmonary antielastase/antimicrobial peptide. The findings support and advance the concept that gene therapy may be used to obviate acute lung injury generally and that caused by bacterial infection specifically by manipulation of host defense molecules (22–26).

Our data indicate that Ad-elafin is capable of protecting A549 cells against acute injury mediated by HNE (Fig. 2). Untransfected A549 cells constitutively produce small quantities of antielastases, including elafin (36), but these were overwhelmed by the concentrations of HNE applied here (Fig. 2*b*), resulting in cell damage. In this context, it is interesting both that a similar concentration of HNE may be found in sputum from patients with CF (3) and that

Table I. Ad-elafin administration is not associated with a significant rise in neutrophil count or protein concentration in BALF^a

	n	White Blood Cell Count ($\times 10^5$)	Neutrophil Count ($\times 10^5$)	Protein in BALF (g/L)	PAO1 Colonies
AD-elafin/PBS	4	2.28 (1.74–3.60)	0.015 (0–0.023)	0.29 (0.25–0.33)	0
AD-lacZ/PBS	4	1.30 (1.02–2.34)	0.045 (0.041–0.060)	0.45 (0.33–0.58)	0
PBS/PBS	4	1.46 (0.90–2.54)	0.011 (0.003–0.026)	0.38 (0.22–0.46)	0

^a C57BL/6 mice received an i.t. dose of one of the following: Ad-elafin (3×10^7 PFU); Ad-lacZ (3×10^7 PFU); or PBS. Five days later, PBS was given i.t. The following day the mice were sacrificed, and BALF was retrieved. The total number of white blood cells in BALF was determined, and the neutrophil count was derived by differential cell counts in cytopins. One aliquot of BALF was cultured overnight to determine the number of *P. aeruginosa* PAO1 colonies (indicating commensal or contaminating bacteria), and another was centrifuged and used to determine protein concentration. Results represent medians and interquartile ranges.

vector per se did not significantly influence outcome in mice receiving bacteria, given that PBS-PAO1 mice had a neutrophil count in BALF of 8.2×10^6 (~200 times the number in mice treated with vector then PBS (Table I)) and a protein concentration of 5.7 g/L (~15 times the concentration in mice treated with vector then PBS (Table I and Fig. 4)). We are not aware of studies demonstrating significant protective effects after doses of adenovirus as low as those used here. Therefore, it appears that gene therapy protocols using sufficiently powerful promoters (17, 18) and low concentrations of vector may, at least in part, be able to circumvent important concerns relating to adenovirus-mediated immune responses (40, 41) and inefficiency of transfection of surface epithelium in the lung (42). Recent advances in vectorology are also likely to improve adenoviral gene delivery to the airways (43).

In our model of murine *P. aeruginosa* lung injury, lungs from control mice showed patchy multilobar consolidation, in keeping with histological appearances in human pneumonia associated with this pathogen (Fig. 4). The appearances were associated with a rise in BALF protein, in turn reflecting disruption of the alveolar-capillary membrane (44, 45). The significant protection against

lung injury consequent on Ad-elafin transfection, as demonstrated by BALF protein levels, was also associated with significantly enhanced elimination of bacteria from the airways (Fig. 4) and with a significantly lower incidence of hematogenous bacterial dissemination. Elafin therefore appears to be part of a network of endogenous pulmonary antibiotics which includes the defensins (14, 16, 46).

The neutrophil may potentially have advantageous or detrimental effects in pulmonary inflammation (Ref. 47; reviewed in Ref. 48). Excessive activation of neutrophils certainly appears to be associated with the potential for tissue damage (48). In the mice studied here, the presence of MPO in BALF provided evidence of neutrophil degranulation and by implication neutrophil activation (Table II). Elafin augmentation was associated with a modest reduction in BALF MPO, potentially supporting the inhibition of neutrophil-mediated damage seen in vitro (Table II and Fig. 3). Elafin augmentation was also associated with a reduction in the neutrophil chemokine mKC, and with a corresponding reduction in total lung neutrophils (Table II). Importantly, the relative reduction in pulmonary neutrophilia associated with elafin augmentation did

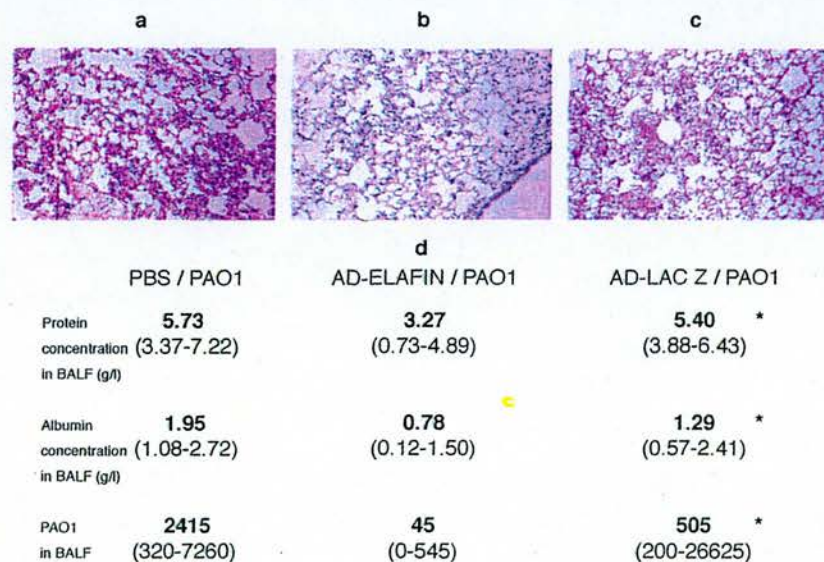


FIGURE 4. Ad-elafin transfection confers protection against acute lung injury induced by *P. aeruginosa* in vivo. C57BL/6 mice were given an i.t. dose of one of the following: Ad-elafin (3×10^7 PFU); Ad-lacZ (3×10^7 PFU); or PBS. Five days later, *P. aeruginosa* PAO1 was given i.t. (34 μ l of a suspension of $\sim 2.2 \times 10^{11}$ CFU/ml). After 24 h, the mice were sacrificed, and BALF was retrieved. One aliquot of BALF was cultured overnight to determine the number of *P. aeruginosa* PAO1 colonies, and another was centrifuged and used to determine protein and albumin concentrations. In a variation of this experiment, lungs were removed en bloc, fixed in formalin, and examined histologically. *a*, Histological appearance of lung from a representative mouse treated with PBS and then *P. aeruginosa* PAO1; *b*, histological appearance of lung from a representative mouse treated with Ad-elafin and then *P. aeruginosa* PAO1; *c*, histological appearance of lung from a representative mouse treated with Ad-lacZ then *P. aeruginosa* PAO1; *d*, protein concentration in BALF, albumin concentration in BALF, and *P. aeruginosa* PAO1 colonies in BALF (results represent medians and interquartile ranges). *, Significant difference by Kruskal-Wallis test, $p < 0.05$: when comparing groups directly, the difference between Ad-elafin/PAO1 and PBS/PAO1 was significant, $p < 0.05$; Ad-elafin/PAO1 was significantly different from Ad-lacZ/PAO1 with respect to protein and PAO1 colonies ($p < 0.05$) but not albumin concentration; no significant differences were observed comparing Ad-lacZ/PAO1 and PBS/PAO1.

Table I. Ad-elafin administration is not associated with a significant rise in neutrophil count or protein concentration in BALF^a

	<i>n</i>	White Blood Cell Count ($\times 10^5$)	Neutrophil Count ($\times 10^5$)	Protein in BALF (g/L)	PAO1 Colonies
AD-elafin/PBS	4	2.28 (1.74–3.60)	0.015 (0–0.023)	0.29 (0.25–0.33)	0
AD-lacZ/PBS	4	1.30 (1.02–2.34)	0.045 (0.041–0.060)	0.45 (0.33–0.58)	0
PBS/PBS	4	1.46 (0.90–2.54)	0.011 (0.003–0.026)	0.38 (0.22–0.46)	0

^a C57BL/6 mice received an i.t. dose of one of the following: Ad-elafin (3×10^7 PFU); Ad-lacZ (3×10^7 PFU); or PBS. Five days later, PBS was given i.t. The following day the mice were sacrificed, and BALF was retrieved. The total number of white blood cells in BALF was determined, and the neutrophil count was derived by differential cell counts in cytopins. One aliquot of BALF was cultured overnight to determine the number of *P. aeruginosa* PAO1 colonies (indicating commensal or contaminating bacteria), and another was centrifuged and used to determine protein concentration. Results represent medians and interquartile ranges.

vector per se did not significantly influence outcome in mice receiving bacteria, given that PBS-PAO1 mice had a neutrophil count in BALF of 8.2×10^6 (~200 times the number in mice treated with vector then PBS (Table I)) and a protein concentration of 5.7 g/L (~15 times the concentration in mice treated with vector then PBS (Table I and Fig. 4)). We are not aware of studies demonstrating significant protective effects after doses of adenovirus as low as those used here. Therefore, it appears that gene therapy protocols using sufficiently powerful promoters (17, 18) and low concentrations of vector may, at least in part, be able to circumvent important concerns relating to adenovirus-mediated immune responses (40, 41) and inefficiency of transfection of surface epithelium in the lung (42). Recent advances in vectorology are also likely to improve adenoviral gene delivery to the airways (43).

In our model of murine *P. aeruginosa* lung injury, lungs from control mice showed patchy multilobar consolidation, in keeping with histological appearances in human pneumonia associated with this pathogen (Fig. 4). The appearances were associated with a rise in BALF protein, in turn reflecting disruption of the alveolar-capillary membrane (44, 45). The significant protection against

lung injury consequent on Ad-elafin transfection, as demonstrated by BALF protein levels, was also associated with significantly enhanced elimination of bacteria from the airways (Fig. 4) and with a significantly lower incidence of hematogenous bacterial dissemination. Elafin therefore appears to be part of a network of endogenous pulmonary antibiotics which includes the defensins (14, 16, 46).

The neutrophil may potentially have advantageous or detrimental effects in pulmonary inflammation (Ref. 47; reviewed in Ref. 48). Excessive activation of neutrophils certainly appears to be associated with the potential for tissue damage (48). In the mice studied here, the presence of MPO in BALF provided evidence of neutrophil degranulation and by implication neutrophil activation (Table II). Elafin augmentation was associated with a modest reduction in BALF MPO, potentially supporting the inhibition of neutrophil-mediated damage seen in vitro (Table II and Fig. 3). Elafin augmentation was also associated with a reduction in the neutrophil chemokine mKC, and with a corresponding reduction in total lung neutrophils (Table II). Importantly, the relative reduction in pulmonary neutrophilia associated with elafin augmentation did

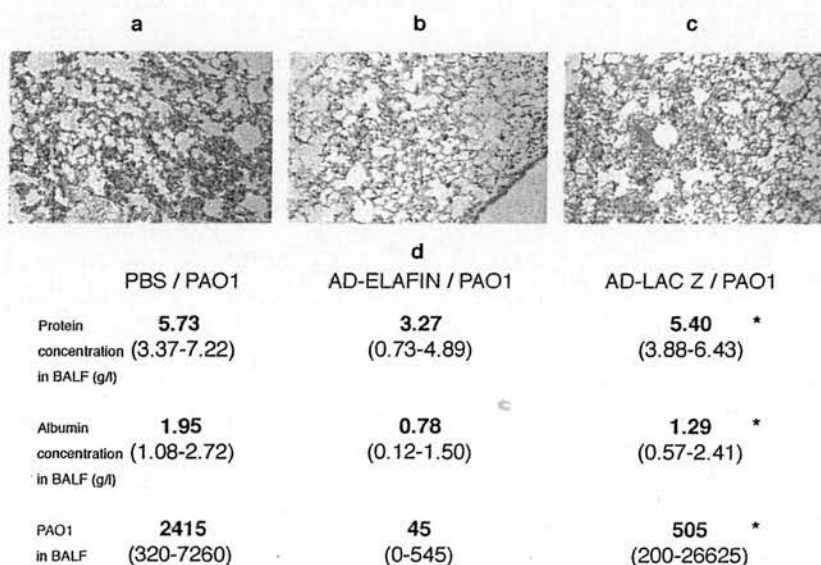


FIGURE 4. Ad-elafin transfection confers protection against acute lung injury induced by *P. aeruginosa* in vivo. C57BL/6 mice were given an i.t. dose of one of the following: Ad-elafin (3×10^7 PFU); Ad-lacZ (3×10^7 PFU); or PBS. Five days later, *P. aeruginosa* PAO1 was given i.t. (34 μ l of a suspension of $\sim 2.2 \times 10^{11}$ CFU/ml). After 24 h, the mice were sacrificed, and BALF was retrieved. One aliquot of BALF was cultured overnight to determine the number of *P. aeruginosa* PAO1 colonies, and another was centrifuged and used to determine protein and albumin concentrations. In a variation of this experiment, lungs were removed en bloc, fixed in formalin, and examined histologically. a, Histological appearance of lung from a representative mouse treated with PBS and then *P. aeruginosa* PAO1; b, histological appearance of lung from a representative mouse treated with Ad-elafin and then *P. aeruginosa* PAO1; c, histological appearance of lung from a representative mouse treated with Ad-lacZ then *P. aeruginosa* PAO1; d, protein concentration in BALF, albumin concentration in BALF, and *P. aeruginosa* PAO1 colonies in BALF (results represent medians and interquartile ranges). *, Significant difference by Kruskal-Wallis test, $p < 0.05$: when comparing groups directly, for each parameter, the difference between Ad-elafin/PAO1 and PBS/PAO1 was significant, $p < 0.05$; Ad-elafin/PAO1 was significantly different from Ad-lacZ/PAO1 with respect to protein and PAO1 colonies ($p < 0.05$) but not albumin concentration; no significant differences were observed comparing Ad-lacZ/PAO1 and PBS/PAO1.

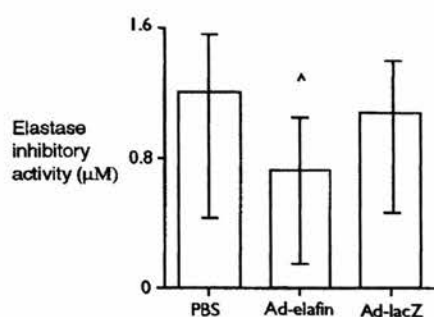


FIGURE 5. Protection against *P. aeruginosa* PAO1 conferred by Ad-elafin is associated with a reduction in EIA. BALF obtained from the mice described in Fig. 4 was analyzed for EIA. EIA was determined by adding sample (serially diluted in buffer (Tris 50 mM, 0.1% Triton, 0.5 M sodium chloride, pH 8)) or buffer alone to a known quantity of purified HNE then adding *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide and measuring change of OD₄₀₅. Plots of OD (reflecting HNE activity) against concentration of sample were constructed. Extrapolation of the curve to the abscissa determined the EIA of each sample. Values represent medians, with bars indicating interquartile ranges. *, Significant difference comparing treatment with Ad-elafin then *P. aeruginosa* PAO1, and treatment with PBS then *P. aeruginosa* PAO1, $p < 0.05$.

not preclude tissue protection, despite the observation that neutrophilia correlates with reduced mortality in pulmonary infection caused by *Nocardia* (47).

Interestingly, no free murine elastase activity was detected in BALF from any of the mice studied. This can be attributed to the characteristic elevation of EIA consequent on severe lung injury, comprising part of the acute phase response (49). We calculate that human elafin contributed less than 1% of the EIA of BALF in mice receiving Ad-elafin. Taken together, these findings suggest that a direct anti-neutrophil elastase effect does not explain the protective effect of Ad-elafin, in keeping with the growing body of evidence that pulmonary anti-neutrophil elastases harbor several functions in addition to inhibition of neutrophil elastase (14–16, 50–53). Furthermore, inhibition of *Pseudomonas* elastase (a metalloelastase) does not explain the effects of Ad-elafin in this study, because elafin is an inhibitor of serine proteinases and not metalloproteinases. Against this background, the reduction in EIA associated with Ad-elafin administration (Fig. 5) reflects a significant and appropriate down-regulation in the acute phase response as a consequence of tissue protection conferred by Ad-elafin. Thus, the protective anti-inflammatory effect of elafin is associated with antimicrobial activity, with a reduction in neutrophils in lung tissue, and with a reduction in the characteristic host response to tissue injury.

The relative level of protection provided by Ad-elafin in our in vivo studies is worthy of note. Ad-elafin effected a 40% reduction

in BALF protein concentrations as compared with control mice (Fig. 4). Given both the severity of injury in the control mice and the remarkable complexity and redundancy in pulmonary anti-inflammatory responses (6), the degree of protection after one administration of a single human gene is encouraging. Interestingly, a nonsignificant trend also emerged potentially suggesting a weak protective effect for adenovirus over vehicle alone (indeed the Ad-lacZ group was included specifically to control for confounding effects of adenovirus). This was manifest as a trend toward lower levels of IL-8 secretion and greater with cytoprotection (Fig. 2d) in vitro and reduction in albumin concentration and EIA in vivo (Figs. 4 and 5). However, the effect seemed most pronounced with regard to bacterial loads, with strikingly (although not statistically significantly) lower levels of bacteria in BALF (Fig. 4) and blood in Ad-lacZ/PAO1 mice as compared with PBS/PAO1 mice. Although it must be emphasized that these trends did not reach statistical significance (whereas differences between Ad-elafin/PAO1 mice and Ad-lacZ/PAO1 mice generally did attain significance), it is tempting to speculate that adenoviral treatment per se may have contributed in some way to bacterial eradication by priming innate immunity. A similar trend has been observed in studies in which rats received sequential i.t. administrations of adenovirus encoding FcγR and *P. aeruginosa* PAO1; delivery of control adenovirus (a null adenovirus, expressing no foreign transgene) resulted in greater clearance of *Pseudomonas* than did PBS, thus further suggesting stimulation of innate immunity by adenovirus per se (54). Interestingly, stimulation of adaptive immunity by recombinant null adenoviruses has also been recognized (55), and priming of immune mechanisms has also been attributed to Ad-lacZ in gene therapy protocols directed at lung tumors (56).

Overall, our data provide proof of principle for the prevention of inflammatory lung injury using augmentation of elafin, particularly in the context of infection with antibiotic-resistant pathogens. *P. aeruginosa* PAO1 is classically associated with antibiotic resistance, and the mechanisms responsible have been extensively characterized (21, 32). More specifically, the approach we describe may potentially find application in preventing colonization with *P. aeruginosa* in CF and in the setting of patients at risk of pneumonia attributable to *P. aeruginosa* in intensive care units (57). Nosocomial *Pseudomonas* pneumonia is associated with a high mortality despite conventional treatment, due in part to antibiotic resistance (21, 58). Current opinion suggests that endogenous cationic peptide antibiotics, such as elafin, may be less susceptible to resistance than are conventional antibiotics (59).

In summary, genetic augmentation of elafin proved effective at protecting pulmonary epithelium against neutrophil-mediated injury in vitro and against acute injury induced by *P. aeruginosa* in vivo. These data support the concept that endogenous defense molecules can contribute to innate immunity by protecting tissue

Table II. Administration i.t. of Ad-elafin is associated with a trend toward reduced pulmonary neutrophilia^a

	PBS/PAO1	Ad-Elafin/PAO1	Ad-lacZ/PAO1
mKC (ng/ml)	0.25 (0.04–0.78)	0.15 (0.02–0.33)*	0.34 (0.27–1.00)
Lung MPO (U/mg wet weight)	577 (314–744)	309 (104–797)	625 (326–804)
Free MPO in BALF (U)	1.13 (0.45–2.28)	0.86 (0.35–1.33)	1.09 (0.31–1.93)

^a Whole lungs and BALF were obtained from the mice described in Fig. 4. MPO content of BALF was assessed by adding the chromogenic substrate tetramethylbenzidine and measuring the change in OD₆₃₀ as a function of time. The left lung of each animal was retrieved, weighed, and snap frozen. On thawing, lungs were immediately homogenized, and MPO content was assessed as described for that in BALF. In addition, mKC levels in BALF were measured by ELISA. Values represent medians, with interquartile ranges in parentheses.

*, Significant difference comparing group treated with Ad-elafin and then *P. aeruginosa* PAO1 and the group treated with Ad-lacZ and then *P. aeruginosa* PAO1; $p < 0.05$.

against microbial damage in vivo, and in so doing they suggest novel therapeutic strategies.

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Regulation of Adenovirus-Mediated Elafin Transgene Expression by Bacterial Lipopolysaccharide

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ABSTRACT

Lipopolysaccharide (LPS) is a mediator of inflammatory lung injury. Selective augmentation of host defense molecules such as elafin (an elastase inhibitor with antimicrobial activity) at the onset of pulmonary inflammation is an attractive potential therapeutic strategy. The aim of this study was to determine whether elafin expression could be induced by LPS administered after transfection with adenovirus (Ad) encoding human elafin downstream of the murine cytomegalovirus (CMV) promoter (known to be potentially responsive to LPS). In addition, we aimed to determine the effect of local elafin augmentation on neutrophil migration to the lung. LPS significantly up-regulated elafin expression from pulmonary epithelial cells transfected with Ad-elafin *in vitro*. In murine airways expression of human elafin was achieved using doses low enough (3×10^7 plaque forming units) to circumvent overt vector-induced inflammation. LPS significantly up-regulated human elafin secretion in murine airways treated with Ad-elafin [117 ng/ml in bronchoalveolar lavage fluid (BALF) after LPS administration, 5.9 ng/ml after PBS, $p < 0.01$]. Over-expression of elafin significantly augmented LPS-mediated neutrophil migration into the airways *in vivo* (1.30×10^6 neutrophils in BALF after Ad-elafin/LPS treatment, 0.54×10^6 after Ad-lacZ/LPS ($p < 0.05$), 0.63×10^6 after PBS/LPS ($p < 0.05$)) and significantly enhanced human neutrophil migration *in vitro*. These data suggest novel functions for elafin in neutrophil migration, and that judicious selection of promoters may allow single, low-dose adenoviral administration to effect inflammation-specific expression of potentially therapeutic transgenes.

OVERVIEW SUMMARY

In designing potential therapies for acute pulmonary inflammatory diseases, a desirable aim would be compartmentalized augmentation of host defense molecules induced specifically by inflammatory mediators. This study demonstrates that transfection with adenovirus encoding human elafin (a low-molecular-weight inhibitor of elastase) downstream of a powerful, lipopolysaccharide (LPS)-responsive promoter (the mCMV promoter) results in elafin secretion which is significantly up-regulated in the presence of LPS, both *in vitro* and *in vivo*. The strength of the promoter used was such that elafin secretion was achieved using doses of adenovirus low enough to obviate overt vector-induced inflammation. This strategy also defined a novel function for elafin, namely enhancement of neutrophil recruitment associated with LPS administration. Therefore, the potential

exists to design gene therapy vectors offering minimization of vector dose and disease-specific up-regulation of transgene in the acute setting.

INTRODUCTION

GENE THERAPY PROTOCOLS are increasingly being applied in models of acute inflammatory lung injury (Greenberger *et al.*, 1996; Bals *et al.*, 1999; Kolls *et al.*, 1999; Chen *et al.*, 2000). An attractive strategy in this context is the therapeutic augmentation of host defense molecules, such as the endogenous pulmonary anti-elastases alpha-1 protease inhibitor (α -1 PI), secretory leukocyte protease inhibitor (SLPI), and elafin [also known as elastase-specific inhibitor (ESI) or skin-derived antileukoprotease (SKALP)] (reviewed by Sallenave, 2000). These molecules are potent inhibitors of human neutrophil elas-

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ase (HNE), a proteolytic enzyme implicated in the pathogenesis of acute lung injury, and found in excess in bronchoalveolar lavage fluid (BALF) or sputum in inflammatory conditions such as cystic fibrosis (CF), acute bacterial pneumonia, non-CF bronchiectasis and emphysema (Gadek *et al.*, 1981; Stockley *et al.*, 1984; Birrer *et al.*, 1994; Boutten *et al.*, 1996).

Elafin is a cationic, low-molecular-weight (10kDa) inhibitor of HNE and proteinase 3, which also has intrinsic antimicrobial activity against bacterial respiratory pathogens (Wiedow *et al.*, 1990, 1991; Sallenave and Ryle, 1991; Sallenave and Silva, 1993; Molhuizen *et al.*, 1993; Schalkwijk *et al.*, 1999; Simpson *et al.*, 1999). Secretion of elafin from airway epithelial cells is rapidly and significantly up-regulated by early inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor- α (TNF- α) (Sallenave *et al.*, 1994). Therefore, elafin appears to constitute part of the lung's innate immune defense. As such, augmentation of elafin could potentially be advantageous in conditions where inflammatory tissue destruction is consequent on bacterial infection. Bacterial lipopolysaccharide (LPS) exerts a number of effects in the generation of acute inflammation and is likely to be a key player in the pathogenesis of sepsis, shock and acute respiratory distress syndrome. The role of LPS in inducing airway neutrophilia and tissue injury has been intensively investigated (Morrison and Ryan, 1987; van Helden *et al.*, 1997).

In this setting, we postulated that LPS may influence elafin secretion from human airway epithelial cells and macrophages. Furthermore, we have previously established adenoviral constructs encoding human elafin cDNA downstream of the powerful murine cytomegalovirus (mCMV) promoter, which is known to contain LPS-responsive elements (Löser *et al.*, 1998). We postulated that such vectors may allow induction of elafin in the presence of LPS. A strategy of this nature could be advantageous under conditions where significant transgene expression is of benefit during episodes of acute inflammation, but undesirable in health. Finally, because antiproteases have been shown to exert various influences on pulmonary neutrophil recruitment (Stockley *et al.*, 1990; Aoshiba *et al.*, 1993; Mackarel *et al.*, 1999), we aimed to determine the effects of elafin augmentation on LPS-induced airway neutrophilia.

METHODS

Cell types

A549 cells were available in-house. A549 cells share several morphological and functional characteristics with type II alveolar epithelial cells (Lieber *et al.*, 1976; Sallenave *et al.*, 1994; van Helden *et al.*, 1999).

Fresh citrated whole blood was obtained from healthy volunteers. Neutrophils and mononuclear cells were derived using a process of dextran (Pharmacia, Uppsala, Sweden) sedimentation and Percoll (Pharmacia) gradient extraction, as described in detail elsewhere (Haslett *et al.*, 1985).

Human macrophages were obtained by plating mononuclear cells on plastic wells (Falcon, Becton Dickinson, Franklin Lakes, NJ) at a density of 4×10^6 /ml in serum-free Iscove's medium (Gibco BRL, Paisley, UK) containing penicillin G (fi-

nal concentration 100 U/ml; Gibco) and streptomycin sulfate (final concentration 100 μ g/ml; Gibco) and incubating at 37°C. After 1 hr, nonadherent cells were washed away and Iscove's medium (containing 10% heat-inactivated autologous serum) was added. Medium was replenished at 3 days. Under these conditions, differentiation to a macrophage phenotype is recognised at 5 days (Wright *et al.*, 1983).

Adenoviral constructs

Two E1-partially E3-deleted type 5 adenoviruses were used. The first (Ad-elafin) comprised human elafin cDNA downstream of a 1.4 kb fragment of the mCMV immediate early (IE) promoter (Dorsch-Häslar *et al.*, 1985). Full-length human elafin cDNA (538 bp) was derived from total RNA extracted from the bronchial epithelial cell line NCI-H322 (Sallenave *et al.*, 1998). The second vector (Ad-lacZ) contained the lacZ reporter gene, again cloned downstream of the 1.4-kb mCMV IE promoter (Addison *et al.*, 1997). Detailed accounts of methods used to generate these constructs are described elsewhere (Addison *et al.*, 1997; Sallenave *et al.*, 1998). In brief, Ad-elafin was isolated by cloning elafin cDNA into plasmid pDK6, which was used along with pBHG10 to co-transfect 293 cells with resultant production of replication-deficient virus (Sallenave *et al.*, 1998). The Ad-lacZ construct was originally created by Addison *et al.* (1997) by cloning lacZ DNA into pMH5, and the resulting plasmid (pCA35) was used, along with pBHG10, to co-transfect 293 cells.

With regard to vector purification and titering, briefly, crude 293 cell lysates were used to infect 293N3S cells in spinner culture flasks. After 3–4 days, the presence of inclusion bodies was typically observed in more than 90% of cells. Cells were pelleted, lysed, and submitted to two consecutive purifications ("banding") using step/continuous CsCl gradients. The adenovirus band was collected and further desalted using PD-10 column chromatography (Pharmacia). Titration (in PFU) of the virus preparations was done in 293 cells using standard techniques (Graham and Prevec, 1991). The optical density (at 260 nm) of the vector preparations was measured, and the estimated number of particles per milliliter was calculated using the formula $OD_{260nm} \times \text{dilution} \times 10^{12}$.

The Ad-elafin preparation was estimated to contain 1.8×10^{10} PFU/ml and 2.4×10^{12} particles/ml (particle:PFU ratio ~133). The Ad-lacZ preparation was estimated to contain 2.0×10^{10} PFU/ml and 2.8×10^{12} particles/ml (particle:PFU ratio ~140).

In vitro experiments—effect of LPS on transfected cells

A549 cells were grown to confluence in 24-well plates (Falcon) using A549 medium, comprising Dulbecco's modified Eagle's medium (DMEM; Sigma, Poole, UK) with 10% fetal calf serum (FCS; Sigma), penicillin G (final concentration 100 U/ml), and streptomycin sulfate (final concentration 100 μ g/ml), at 37°C and 5%CO₂ in a humidified incubator. Cells were washed with phosphate-buffered saline (PBS; Sigma). A549 medium (modified such that the FCS concentration was 5%) with or without virus construct at a multiplicity of infection (MOI) of 1 plaque forming unit (PFU) was then added for 30 min at 37°C (final volume 50 μ l). Cells were washed with PBS to remove excess

virus, and 400 μ l of A549 medium was added. After 48 hours 40 μ l of A549 medium containing LPS (from *Escherichia coli* 0127:B8; Difco Laboratories, Detroit, MI, USA) was added (final concentration of LPS 0, 0.1, 1, 10, or 100 μ g/ml). Cells were incubated at 37°C for 24 hr and supernatants retrieved and frozen at -80°C until further use.

Human monocyte-derived macrophages were washed with PBS then treated for 30 min at 37°C with Iscove's medium containing 5% autologous serum, with or without virus construct at an MOI of 50 PFU. Cells were washed with PBS to remove residual virus, and then incubated with 400 μ l of Iscove's medium containing 10% heat-inactivated autologous serum for 48 hr. LPS was serially diluted in Iscove's medium containing 10% autologous serum, and 40- μ l aliquots added directly (final concentration of LPS 0, 0.1, 1, 10, or 100 μ g/ml). The cells were incubated for 24 hr at 37°C. Cell supernatants were retrieved and frozen at -80°C until further use.

Preliminary experiments demonstrated that application of LPS in the dose range 0.1–1000 μ g/ml did not significantly alter viability of untransfected or adenovirus-transfected A549 cells or macrophages (data not shown).

Transfection efficiency of A549 cells and monocyte-derived macrophages was determined by infecting with Ad-lacZ as described above, and incubating for 48 hr at 37°C. Medium was discarded and 200 μ l of fixative (0.2% glutaraldehyde, 0.8% formaldehyde, 2 mM MgCl₂ in PBS; all Sigma) added for 10 min at room temperature. Fixative was discarded and 200 μ l of staining solution (5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 2 mM MgCl₂, 0.05% Triton X-100, 0.5 mg/ml X-gal in PBS; all Sigma except for X-gal [Promega, Madison, WI]) added for 5 hr at 37°C. Blue staining was taken to be indicative of transgene expression as a marker of transfection efficiency.

In vitro neutrophil migration experiments

A549 cells were grown to confluence in 24-well plastic plates (Costar, Cambridge, MA) and treated with Ad-elafin (MOI of 50 PFU), Ad-lacZ (MOI of 50 PFU), or medium alone as described above, with the exceptions that Phenol Red-free DMEM (PRF-DMEM; Sigma) was used, and that medium used after transfection of A549 cells was serum-free. The volume of medium in each well was 500 μ l. After 48 hr, fresh human neutrophils were prepared and suspended in PRF-DMEM containing 1% bovine serum albumin (BSA; Sigma) at a concentration of 12.5×10^6 neutrophils/ml. BSA was simultaneously added to the A549 cell supernatants (1% final concentration). A total of 100 μ l of the neutrophil suspension was added to a porous polycarbonate filter insert with a pore size of 3 μ m (Costar). Each insert was immediately placed into the wells containing A549 cells such that the insert base was in contact with the supernatant, and incubated for 20 min at 37°C, after which inserts were removed and discarded. Lysis of neutrophils that had migrated through the filter was then achieved by addition of Triton X-100 (Sigma) (final concentration 0.4%). The resulting lysate was acidified using 12.5 μ l of sodium citrate-acetate buffer (pH 4.2; Sigma). Myeloperoxidase (MPO) activity was used as an index of neutrophil content and was measured by adding MPO-specific chromogenic substrate (0.1 mg/ml tetramethylbenzidine (TMB; Boehringer Mannheim, Mannheim, Ger-

many), 0.03% H₂O₂ (Sigma), in 0.1 M sodium acetate-citrate (pH 4.9; Sigma), and quantifying the change in absorbance at 630 nm using a Dynatech M5000 Plate Reader (Dynex, Billingham, West Sussex, UK). The number of neutrophils in lysates was estimated by extrapolation from standard curves prepared by lysing known quantities of neutrophils and plotting against the change in absorbance at 630 nm after addition of TMB and H₂O₂. Because neutrophils from different donors varied in their rate of maximal migration (range 89,000–299,000; median 237,000), neutrophil migration was expressed relative to maximal migration for each experiment. In a separate experiment, an excess of polyclonal rabbit anti-human-elafin IgG was incubated with cells at 37°C prior to the addition of neutrophils.

In vivo experiments

Female C57/Bl6 mice aged between 6 and 8 weeks were from Harlan Olac (Bicester, UK). All treatments were performed under anesthesia using intraperitoneal avertin (1.25% 2,2,2-tribromoethanol, Aldrich, Gillingham, Dorset, UK; 2.5% 2-methyl-2-butanol, Sigma) at approximately 10 μ l per gram of body weight. All treatments were delivered by intratracheal (IT) instillation, using a total volume of 30 μ l administered under direct visualisation of the larynx (McLachlan *et al.*, 2000). In all experiments, after the required series of instillations, mice were sacrificed under anesthesia by transection of the abdominal aorta. The lungs and trachea were removed *en bloc*, and bronchoalveolar lavage was immediately performed by serial instillation of 250 μ l then 200 μ l of PBS. BALF was centrifuged at 2000 rpm for 10 minutes at 4°C and the supernatant stored at -80°C. The remaining pellet was resuspended in fresh PBS, and an aliquot taken for measurement of total cell count using a hemacytometer. A separate aliquot was used to prepare cytopins at 300 rpm for 3 min at room temperature. Cytopins were stained with Diff-quick (Dade Diagnostika, Germany) for analysis of differential cell count.

Lungs were fixed in formalin (Sigma) and embedded in paraffin wax. Sections 3 μ m thick were cut, stained with hematoxylin and eosin, and photomicrographs were taken.

In a preliminary experiment aiming to establish the optimal dose of adenoviral vector, a single IT instillation of Ad-elafin at either 3×10^7 PFU or 3×10^8 PFU was given, and mice sacrificed 3, 6, or 8 days after instillation. In a separate preliminary experiment aiming to establish a dose of LPS producing a moderate (approximately 50%) pulmonary neutrophilia at 24 hr, a single IT instillation of either 0.1 μ g or 0.5 μ g of LPS was given, and mice were sacrificed 24 hr later. *E. coli* 0127:B8 LPS was used in all *in vivo* experiments. As described in detail later, Ad-elafin at a dose of 3×10^7 PFU consistently generated human elafin in murine airways without a significant increase in inflammatory cells in the airways, whereas 0.5 μ g of LPS produced a moderate neutrophilia (Table 1).

Therefore, in subsequent experiments either PBS, or 3×10^7 PFU of Ad-lacZ, or 3×10^7 PFU of Ad-elafin were given IT ($n = 12$ in each group). Five days later, 4 mice in each group received an IT instillation of PBS and the remaining 8 mice in each group received an IT instillation of 0.5 μ g of LPS. Mice were sacrificed 24 hr later; BALF and lung histology were performed as described above.

In separate experiments, mice were treated IT with 3×10^7 PFU of Ad-lacZ as above, then five days later with either 0.5 μ g or 5 μ g of LPS. The following day lungs were removed *en bloc* and fixed for 60 min at 4°C using 2% formaldehyde containing 0.2% glutaraldehyde. The fixative was washed out with PBS and the lungs stained using 5 mM K₄Fe (CN)₆, 5 mM K₃Fe₃ (CN)₆, 2 mM MgCl₂, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (all Sigma). Sections were taken and counterstained with Nuclear Fast Red (Sigma).

Assays

Elafin concentration was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) performed on 96 well plates (Linbro, Flow Laboratories, McLean, VA). In brief, the primary antibody was polyclonal rabbit anti-human elafin immunoglobulin. Gelatin (BDH, Poole, Dorset, UK) was used as a blocking agent, the sample (or purified recombinant elafin as standard) was applied, and the secondary antibody was biotinylated polyclonal rabbit anti-human elafin immunoglobulin (biotin was from Pierce, Rockford, IL). Streptavidin-horseradish peroxidase complex (Sigma) then chromogenic substrate (2,2'-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid; Sigma]) were added sequentially and absorbance of the product at 550 nm was quantified using a Dynatech M5000 Plate Reader.

IL-8, macrophage inflammatory protein (MIP)-2 and murine TNF- α concentrations were measured using commercial ELISA kits (R&D Systems, Abingdon, Oxon, UK). ELISA for MIP-1 α was kindly performed by Professor T. Standiford (Ann Arbor, MI). Protein levels were measured using the bicinchoninic acid method (Pierce, Rockford, IL).

Statistical analysis

When data were normally distributed Student's *t*-test was used. When data did not conform to a normal distribution, non-parametric tests were used. Three group comparisons were performed using the Kruskal-Wallis test. Paired comparisons were analyzed using Wilcoxon's signed rank test, unpaired comparisons using the Mann-Whitney U-test, and correlations using

Spearman's rank correlation test. Statistical significance was assigned to $p < 0.05$.

RESULTS

Effect of LPS on elafin secretion in vitro

In response to LPS, A549 cells transfected with Ad-lacZ secreted concentrations of elafin similar to those secreted by untransfected A549 cells, indicating that viral transfection *per se* did not significantly influence elafin production (Table 1). However, in A549 cells transfected with Ad-elafin, LPS stimulation resulted in significant up-regulation of elafin production, the magnitude of the augmentation being considerably greater than that seen in untransfected or Ad-lacZ-treated cells (Table 1).

LPS stimulation did not augment elafin levels in supernatants from untransfected human macrophages or macrophages transfected with Ad-lacZ (Table 2). Transfection efficiency of macrophages was approximately 16% indicating that the lack of effect in Ad-lacZ treated cells was not due to absence of transfection. In contrast, LPS appeared to induce a moderate increase in elafin secretion from macrophages transfected with Ad-elafin (Table 2).

Effect of elafin over-expression on neutrophil migration in vitro

Ad-elafin transfection resulted in a significant increase in migration of human neutrophils across a polycarbonate filter of 3- μ m pore size (Fig. 1). This effect was not attributable to induction of IL-8 release, because the median IL-8 concentration in cell supernatants from untransfected cells was 37.2 pg/ml, from Ad-lacZ-treated cells 9.3 pg/ml, and from Ad-elafin-treated cells 23.8 pg/ml ($p = 0.05$ comparing the three groups). Preincubation of cells with anti-elafin antibody reduced neutrophil migration in Ad-elafin-treated cells to levels observed in Ad-lacZ-treated cells (percentage of maximal migration 65% in Ad-elafin-treated cells, 69% in Ad-lacZ-treated cells, and 21% in untransfected cells).

TABLE 1. LPS SIGNIFICANTLY INCREASES SECRETION OF ELAFIN FROM A549 CELLS TRANSFECTED WITH AD-ELAFIN

	Dose of LPS (μ g/ml)				
	0	0.1	1	10	100
Untransfected	3.0 ^a (2.0–4.7)	3.4 ^a (2.6–4.2)	4.8 ^{a,b} (3.3–6.4)	8.4 ^{a,b} (5.9–23.0)	15.0 ^{a,b} (10.5–24.9)
Ad-lacZ	3.1 ^a (2.7–7.2)	3.5 ^a (3.1–10.5)	5.3 ^a (3.8–13.1)	12.5 ^{a,b} (5.2–40.4)	10.5 ^a (6.3–13.8)
Ad-elafin	15.6 (12.4–29.8)	14.8 (10.5–35.4)	20.7 ^b (16.2–37.0)	33.4 ^b (20.5–70.6)	37.6 ^b (23.2–62.6)

A549 cells were treated with medium alone, Ad-lacZ (MOI of 1 PFU), or Ad-elafin (MOI of 1 PFU), then incubated for 48 hr. LPS was added over the concentration range 0–100 μ g/ml for a further 24 h and elafin concentrations measured.

Results represent elafin concentrations (in ng/ml) and are expressed as medians (and interquartile ranges) from five separate experiments.

^a $p < 0.05$ compared with elafin levels from Ad-elafin-treated cells receiving the same dose of LPS.

^b $p < 0.05$ compared with the baseline value (*i.e.*, no addition of LPS) for each given treatment.

TABLE 2. LPS STIMULATES EXPRESSION OF ELAFIN TRANSGENE IN HUMAN MACROPHAGES TRANSFECTED WITH Ad-ELAFIN

	Dose of LPS ($\mu\text{g/ml}$)				
	0	0.1	1	10	100
Untransfected	1.2 (0.8–1.5)	0.9 (0.8–1.1)	0.9 ^a (0.7–1.0)	0.6 ^{a,b} (0.6–0.9)	0.6 ^a (0.6–1.1)
Ad-lacZ	0.7 (0.6–1.8)	0.8 ^a (0.6–1.0)	0.8 ^a (0.7–1.0)	0.7 (0.6–1.3)	0.8 ^a (0.8–1.0)
Ad-elafin	1.3 (0.9–11.6)	1.8 (1.1–24.3)	9.6 (0.9–23.4)	6.0 (0.7–11.1)	6.4 (1.4–23.3)

Human macrophages were treated with medium alone, Ad-lacZ (MOI of 50 PFU), or Ad-elafin (MOI of 50 PFU) and incubated for 48 hr. LPS was added over the concentration range 0–100 $\mu\text{g/ml}$ for a further 24 hr and elafin concentrations were measured.

Results represent elafin concentrations (in ng/ml) and are expressed as medians (and interquartile ranges) from six separate experiments.

^a $p < 0.05$ compared with elafin levels from Ad-elafin-treated cells receiving the same dose of LPS.

^b $p < 0.05$ compared with the baseline value (i.e., no addition of LPS) for each given treatment.

Effect of human elafin over-expression on LPS-induced neutrophilia in murine airways

Preliminary experiments indicated that a low dose (3×10^7 PFU) of Ad-elafin administered IT resulted in significant expression of human elafin in BALF without overt airway neutrophilia attributable to the vector (Table 3). In keeping with this observation, low-dose adenovirus administration resulted in transfection of bronchial and alveolar epithelium *in vivo* (Fig. 2). Higher doses of adenovirus were associated with significant airway inflammation (Table 3). Preliminary data further indicated that moderate airway neutrophilia could be provoked by administering 0.5 μg of LPS IT (mean BALF neutrophil count 170,200, equating to 54.4% of the total cellular content, $n = 2$), but not by 0.1 μg of LPS given IT (mean BALF neutrophil count 47,700, equating to 12.4% of the total cellular content, $n = 2$).

On the basis of these experiments mice were treated with either PBS, 3×10^7 PFU Ad-lacZ, or 3×10^7 PFU Ad-elafin delivered IT. Five days later, mice in these three groups were treated IT with either PBS or 0.5 μg of LPS. Elafin was detected in BALF from all mice initially receiving Ad-elafin, but in none of the mice initially treated with PBS or Ad-lacZ, reflecting the established specificity of our ELISA for human elafin (no murine analogue of elafin has yet been identified; the absence of detectable elafin in the control animals indicates that, should such a molecule exist, it does not cross-react with our anti-human elafin antibody). In mice pre-treated with Ad-elafin, LPS administration resulted in median BALF elafin levels 20 times higher than did PBS administration (Fig. 3A). Further independent evidence for significant activation of the mCMV promoter by inflammatory signals was provided by the LPS-dependent increase in β -galactosidase expression in mice transfected with Ad-lacZ (Fig. 3B).

LPS-induced airway neutrophilia was significantly augmented in mice pretreated with Ad-elafin (Fig. 4A,B), in keeping with our findings *in vitro* (Fig. 1). The augmentation of neutrophilia in Ad-elafin/LPS-treated mice was associated with an increase in both MIP-2 and TNF- α in BALF (Table 4). Among all mice treated with LPS, BALF neutrophil count correlated significantly with MIP-2 concentrations ($r = 0.61$, $p < 0.01$).

In the absence of LPS, adenoviral transfection *per se* did not significantly influence airway neutrophilia, as evidenced by normal neutrophil counts in BALF after adenoviral transfection (Table 3), and the absence of a rise in TNF- α in the airways of mice treated with vector then PBS (median BALF TNF- α levels 405 pg/ml in PBS/PBS mice, 359 pg/ml in Ad-lacZ/PBS mice, and 375 pg/ml in Ad-elafin/PBS mice; no significant difference).

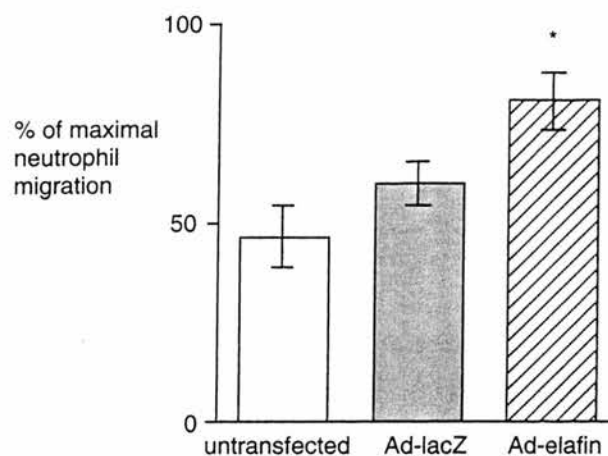


FIG. 1. Elafin augmentation enhances neutrophil migration *in vitro*. A549 cells were treated with medium alone, Ad-lacZ (MOI of 50 PFU), or Ad-elafin (MOI of 50 PFU) and incubated in serum-free medium for 48 hr. Freshly prepared human neutrophils were added to inserts containing 3- μm pores, which were immediately placed into wells containing A549 cells for 20 min and then discarded. Migrating neutrophils were lysed and the MPO content of lysates determined, as an index of neutrophil count. For each of four separate experiments, conditions were performed in quadruplicate. The maximal rate of neutrophil migration was calculated for each experiment, and migration was expressed relative to this value. Results represent means (and standard deviations) of the percentage maximal migration for each treatment. *, $p < 0.05$ compared with both other groups.

TABLE 3. INTRATRACHEAL ADMINISTRATION OF LOW-DOSE ADENOVIRUS RESULTS IN EXPRESSION OF HUMAN ELAFIN WITHOUT INDUCING SIGNIFICANT AIRWAY NEUTROPHILIA

	Day 3	Day 6	Day 8
3×10^7 PFU Ad-elafin			
Neutrophil count (% of total cells)	2300 (1.9%)	900 (1.2%)	1000 (1.1%)
Elafin (ng/ml)	0.6	0.9	4.9
3×10^8 PFU Ad-elafin			
Neutrophil count (% of total cells)	288,200 (31.0%)	256,000 (46.9%)	183,300 (23.4%)
Elafin (ng/ml)	19.5	34.8	6.3

C57/Bl6 mice received IT Ad-elafin (either 3×10^7 PFU or 3×10^8 PFU) and were sacrificed on day 3, day 6, or day 8. Neutrophil and total cell counts were determined in BALF, along with elafin concentration.

Results represent medians; $n = 3$ in each group.

A trend toward increased numbers of pulmonary macrophages was also observed in mice treated with Ad-elafin and then LPS (median counts 257,500 after PBS/LPS treatment, 183,400 after Ad-lacZ/LPS, and 326,600 after Ad-elafin/LPS; no significant difference). This trend was associated with an increase in the concentration of MIP-1 α in BALF from mice receiving Ad-elafin/LPS (Table 4). However, in all groups of mice, the magnitude of the rise in alveolar macrophages was considerably less than that for airway neutrophils after LPS administration (for example, comparing PBS/PBS mice with PBS/LPS mice, macrophages increased 1.8-fold, whereas neutrophils increased over 500-fold).

Despite the augmentation of neutrophil (and to a lesser extent macrophage) numbers in the airways of Ad-elafin/LPS-treated mice, there was no associated enhancement of alveolar-capillary membrane disruption, mean protein concentrations in BALF being 0.51 gram/liter (SD 0.49) in PBS/LPS mice, 0.47 gram/liter (0.50) in Ad-lacZ/LPS mice, and 0.42 (0.29) in Ad-elafin/LPS mice (no significant difference comparing each of the groups).

DISCUSSION

Selective up-regulation of endogenous defence molecules during inflammatory tissue injury is an attractive potential therapeutic goal. It may be especially pertinent to modulate expression of anti-inflammatory molecules known to be deficient in inflammatory conditions, whether that deficiency be hereditary (as in α -1 PI deficiency) (Gadek *et al.*, 1981) or acquired (as in the reduction in airway elafin described in CF and chronic obstructive pulmonary disease) (reviewed by Sallenave, 2000). The data presented here indicate that, in gene therapy protocols, judicious choice of promoters responsive to inflammatory mediators may result in selective induction of transgene expression in pulmonary inflammation. Furthermore, they support the concept that up-regulation of endogenous defense molecules in complex inflammatory conditions may be used to elucidate novel functions, as evidenced by enhanced LPS-mediated neutrophil migration after elafin augmentation.

The observation that LPS up-regulates elafin expression in native (untransfected) pulmonary epithelial cells (Table 1) sug-

gests that the endogenous human elafin promoter may be responsive to exogenous inflammatory stimuli. This extends the body of evidence suggesting that elafin secretion from pulmonary epithelial cells may variously be enhanced, unaltered, or reduced by important endogenous inflammatory mediators (Sallenave *et al.*, 1994; Reid *et al.*, 1999; van Wetering *et al.*, 2000).

Adenoviral transfection of pulmonary epithelial cells did not of itself appear to stimulate elafin release, because application of Ad-lacZ did not stimulate elafin secretion above that from untransfected cells (Table 1). The increase in LPS-induced elafin secretion after low-dose (MOI of 1 PFU) Ad-elafin transfection over and above that from untransfected cells can therefore be attributed to the Ad-elafin vector (Table 1). Furthermore, the up-regulation appears to be due to the effects of LPS on the mCMV promoter at the transcriptional level, because our construct does not contain a 5' untranslated region (Sallenave *et al.*, 1998). The pronounced elafin expression observed despite low dose Ad-elafin transfection confirms the inherent power of the mCMV promoter (Addison *et al.*, 1997; Löser *et al.*, 1998; Sallenave *et al.*, 1998).

In contrast to the findings in pulmonary epithelial cells, LPS stimulation produced modest enhancement of elafin secretion from Ad-elafin treated macrophages, but not from untransfected or Ad-lacZ-transfected macrophages (Table 2). These data suggest that elafin production was derived from the elafin transgene rather than the endogenous elafin gene. In our hands, adenoviral transfection of macrophages was markedly less efficient than that observed in A549 cells, as has been described elsewhere (Kaner *et al.*, 1999).

Transfection occurred at a rate of approximately 16%, in keeping with similarly low rates of transfection reported by others for monocyte-derived macrophages (Haddada *et al.*, 1993; Schneider *et al.*, 1997) and alveolar macrophages (Worgall *et al.*, 1999). However, although adenoviral vectors are rapidly internalized and, to a large extent, cleared by alveolar macrophages *in vivo* (Worgall *et al.*, 1997; Zsengellér *et al.*, 2000), persistence of potentially therapeutic transgene expression has been noted in alveolar macrophages in murine models (Danel *et al.*, 1998; Worgall *et al.*, 1999). We similarly found expression of transgene in occasional alveolar macrophages 6 days after transfection in our LPS-treated mice (data not shown). This emphasizes the point that alveolar protection could potentially

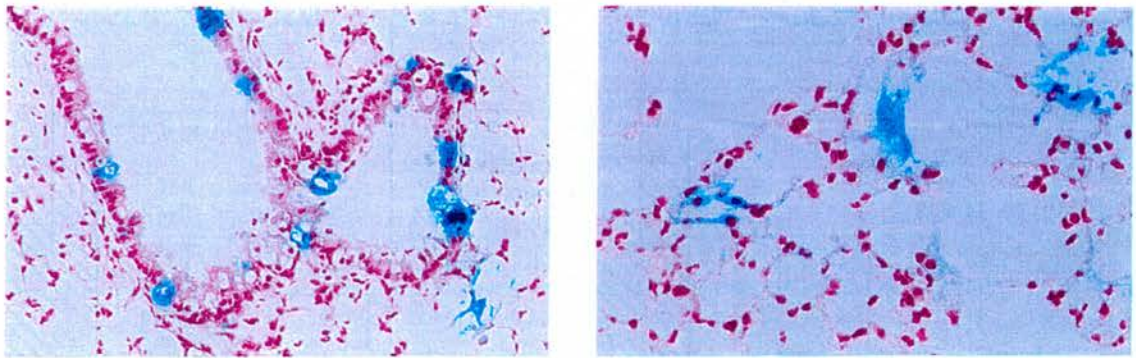


FIG. 2. Intratracheal administration of low-dose adenovirus results in efficient transfection of bronchial and alveolar epithelium. C57/Bl6 mice received Ad-elafin (3×10^7 PFU) IT. Five days later, $0.5 \mu\text{g}$ of LPS was administered IT and mice were sacrificed 24 hr later. Lung sections were stained with X-gal solution. (Left panel) Positive (blue) staining in bronchial epithelium; (right panel) positive staining in alveolar epithelium.

be simultaneously and effectively provided by macrophages and epithelial cells in gene therapy strategies (Danel *et al.*, 1998).

The observation that Ad-elafin and Ad-lacZ transgene expression was significantly augmented in response to LPS in murine airways supports the concept that LPS stimulates the mCMV promoter powerfully *in vivo*, either directly or indirectly (Fig. 3). This suggests potential therapeutic applications whereby expression of transgenes could be induced selectively in the context of inflammation, particularly that involving bacterial products such as LPS. Therefore, our data add the mCMV promoter to the growing list

of promoters that can be activated selectively during inflammation or induced using exogenous activators (Gossen *et al.*, 1995; Varley *et al.*, 1995; No *et al.*, 1996). The strength of the mCMV promoter was such that we were able to achieve significant transgene expression using doses of adenovirus low enough so as not to provoke overt vector-induced neutrophilia (Table 3 and panel a of Fig. 4B). Further evidence for the relative absence of vector-induced inflammation (or contamination of vector by exogenous inflammatory mediators) was provided *in vivo* by lower levels of airway TNF- α in vector-treated mice than in PBS-treated mice,

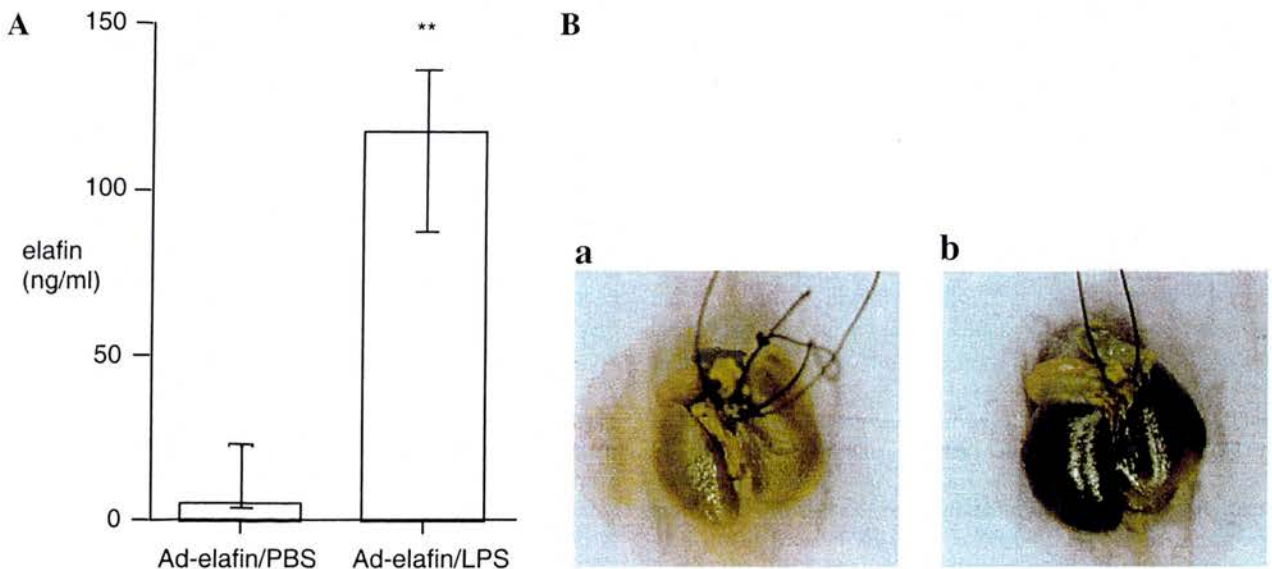


FIG. 3. LPS stimulation significantly increases expression of transgenes under the control of the mCMV promoter in murine lungs. (A) C57/Bl6 mice received IT Ad-elafin (3×10^7 PFU). Five days later, 4 animals received IT PBS, while another 8 received IT LPS ($0.5 \mu\text{g}$). Twenty-four hours later, mice were sacrificed and elafin concentrations measured in BALF. Results represent medians and interquartile ranges. $** p < 0.01$ comparing the two groups. (B) C57/Bl6 mice received IT Ad-lacZ (3×10^7 PFU). Five days later, mice received either $0.5 \mu\text{g}$ or $5 \mu\text{g}$ of LPS IT. Twenty-four hours later, mice were sacrificed and the lungs stained with X-gal solution. (a) Treatment with $0.5 \mu\text{g}$ of LPS. (b) Treatment with $5 \mu\text{g}$ of LPS. A faint blue color was seen after treatment with $0.5 \mu\text{g}$ of LPS, the effect being markedly increased after treatment with $5 \mu\text{g}$ of LPS.

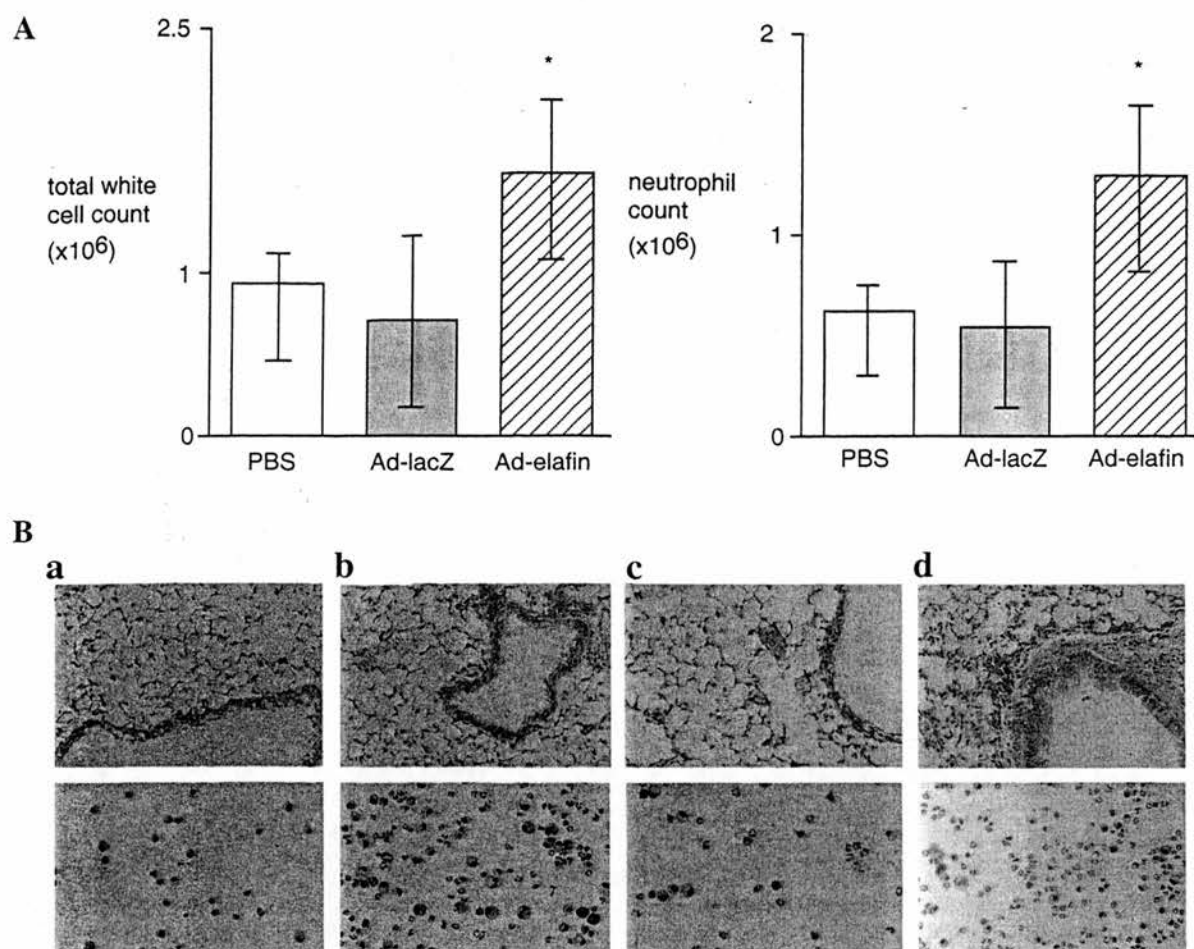


FIG. 4. Transfection of murine airways with Ad-elafin significantly augments LPS-induced airway neutrophilia. **(A)** C57/B16 mice received IT PBS, Ad-lacZ (3×10^7 PFU), or Ad-elafin (3×10^7 PFU) ($n = 8$ in each group). Five days later, mice were given $0.5 \mu\text{g}$ of LPS IT. Twenty-four hours later, animals were sacrificed. Neutrophil and total cell counts were measured in BALF. Results represent medians and interquartile ranges. * $p < 0.05$ compared with both other groups. **(B)** C57/B16 mice were treated as described above until sacrifice, when lungs were stained with hematoxylin and eosin. For each treatment the upper panel illustrates lung histology and the lower panel illustrates cytopins from BALF. **(a)** Mouse receiving Ad-elafin then PBS instead of LPS. **(b)** Mouse receiving PBS then LPS. **(c)** Mouse receiving Ad-lacZ then LPS. **(d)** Mouse receiving Ad-elafin then LPS. In **a**, there is no inflammatory cell infiltrate and the predominant cell type in BALF is the macrophage. In **b** and **c**, there is a moderate cellular infiltrate, with a mixed population of neutrophils and macrophages in BALF. In **d**, there is a marked inflammatory infiltrate, and the number and proportion of neutrophils in BALF is greater than that in **b** or **c**.

and *in vitro* by lower IL-8 levels in vector-treated A549 cells than in PBS-treated cells. As such, selective single administration of adenoviral constructs may, at least in part, obviate concerns relating to vector-induced inflammation (Yang *et al.*, 1994; van Ginkel *et al.*, 1995). The absence of vector-induced airway neutrophilia was particularly important in this study because one of the aims was to determine the effect of human elafin over-expression on LPS-induced neutrophilia.

Interestingly LPS inhibits neutrophil mobility and chemotaxis *in vitro* (Dahinden *et al.*, 1983; Haslett *et al.*, 1985) but causes neutrophil extravasation in tissues such as skin (Cybulsky *et al.*, 1988) and lung (Hudson *et al.*, 1977) after local administration *in vivo*. Clearly, neutrophil chemokines and other

mediators induced by LPS *in situ* could explain this apparent paradox but our data suggest for the first time that elafin may be involved, in that elafin augmentation was associated with significant enhancement of LPS-induced airway neutrophilia *in vivo* (Fig. 4). Neutrophil migration was not enhanced in murine airways exposed to Ad-elafin in the absence of LPS (Table 3); this may suggest that elafin's effect on neutrophil migration is dose-dependent, or that the effect is dependent upon collaboration with LPS or other secondary inflammatory signals. However, it is clear from our *in vitro* data that elafin augmentation also enhances migration of human neutrophils in a system in which LPS is not added (Fig. 1). Although our *in vitro* data suggest that adenoviral transfection *per se* contributes in part

TABLE 4. TRANSFECTION OF MURINE AIRWAYS WITH Ad-ELAFIN AUGMENTS LEVELS OF CYTOKINE PRODUCTION ASSOCIATED WITH LPS ADMINISTRATION

	PBS/LPS	Ad-lacZ/LPS	Ad-elafin/LPS
MIP-2 (pg/ml)	257.4 (224.8–303.3)	121.7 (44.1–234.4)	318.1 ^a (286.2–369.6)
TNF- α (pg/ml)	711	617	1319
MIP-1 α (pg/ml)	188	139	205

C57/Bl6 mice received IT PBS, Ad-lacZ (3×10^7 PFU), or Ad-elafin (3×10^7 PFU) ($n = 8$ in each group, as described in Fig. 4). Five days later, mice received 0.5 μ g of LPS IT. Twenty-four hours later, animals were sacrificed, and MIP-2, MIP-1 α , and TNF- α concentrations were measured in BALF. For TNF- α and MIP-1 α , BALF from mice in each group was pooled to generate sufficient sample.

Results for MIP-2 represent medians (and interquartile ranges).

^a $p < 0.05$ comparing the three groups using the Kruskal-Wallis test. For comparisons between pairs of groups, a significant difference was found comparing Ad-lacZ/LPS and Ad-elafin/LPS, $p < 0.05$.

to enhancement of neutrophil migration, this effect was not observed in our *in vivo* studies.

The mechanism by which elafin augmentation enhances neutrophilia in our models is unclear. Elafin fragments of 6 kDa do not influence neutrophil migration across endothelial barriers *in vitro* (Mackarel *et al.*, 1999), and elafin has been associated with reduced neutrophil recruitment in models of skeletal muscle reperfusion injury and myocardial ischaemia *in vivo* (Crinnion *et al.*, 1994; Tiefenbacher *et al.*, 1997). It should be noted that the studies alluded to used elafin fragments (whereas the whole molecule was generated in our study), and that both *in vivo* studies used the intravenous route in rat models, generating initial circulating concentrations of elafin far in excess of those generated in BALF in this study.

Ad-elafin/LPS administration was associated with increased concentrations of early inflammatory cytokines in BALF (Table 4), raising the possibility that elafin can induce secretion of such molecules either directly or indirectly. However elafin-mediated augmentation of human neutrophil migration *in vitro* was not associated with an elevation of IL-8, one of the main C-X-C neutrophil chemokines.

HNE appears to have a complex role in modulating neutrophil recruitment (Owen *et al.*, 1995; Cai and Wright, 1996; Leavell *et al.*, 1997; le-Barillec *et al.*, 1999), and elastase inhibitors have been shown to have varying effects on neutrophil migration (Stockley *et al.*, 1990; Aoshiba *et al.*, 1993; Mackarel *et al.*, 1999). We cannot exclude the possibility that an interaction between augmented elafin levels and murine neutrophil elastase explained the increase in neutrophil migration (certainly human elafin can inhibit murine elastase; Prof G Lungarella, University of Siena, personal communication). However, it remains distinctly possible that elafin's role in augmenting pulmonary neutrophilia may be unrelated to anti-elastase activity. Elafin shares certain characteristics with the defensins in being a low-molecular-weight cationic molecule with antimicrobial activity. Not only are these characteristics associated with an avidity for LPS (Peterson *et al.*, 1985), but human neutrophil defensin has been associated with neutrophil accumulation in murine models of Gram-negative peritoneal infection (Welling *et al.*, 1998).

Importantly, the doubling of airway neutrophils associated with Ad-elafin/LPS treatment (Fig. 4) was not associated with an increase in protein concentration in BALF, implying preservation of alveolar-capillary membrane integrity. Indeed, median BALF protein concentrations in all three groups of mice treated with LPS approximated to normal in this study, suggesting minimal disruption to the membrane in association with neutrophil influx. In this setting, it is tempting to speculate that elafin may function as an important regulatory molecule in early innate immunity; elafin secreted in response to bacterial LPS may simultaneously provide antimicrobial protection and up-regulate neutrophil influx while protecting host tissue from the injurious effects of extracellular HNE. In this context, it is of considerable interest that SLPI (which shares several characteristics with elafin) has been shown to have a number of functions integral to innate immunity (Hiemstra *et al.*, 1996; Jin *et al.*, 1997; Zhang *et al.*, 1997) and that augmentation of SLPI confers protection in models of pulmonary inflammation (Mulligan *et al.*, 1993; Rudolphus *et al.*, 1993; Gipson *et al.*, 1999).

In summary, gene therapy protocols can be designed such that single, small doses of adenoviral vector generate transgenes selectively expressed in the presence of inflammatory mediators. Such strategies may have important therapeutic consequences. Finally, applications such as those described in this study help to elucidate the functions of transgene products expressed in the lung, as evidenced by the demonstration of elafin's augmentation of LPS-induced neutrophilia.

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Elafin (elastase-specific inhibitor) has anti-microbial activity against Gram-positive and Gram-negative respiratory pathogens

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Abstract Elafin (elastase-specific inhibitor) is a low molecular weight inhibitor of neutrophil elastase which is secreted in the lung. Using synthetic peptides corresponding to full-length elafin (H₂N-¹AVT.....⁹⁵Q-OH), the NH₂-terminal domain (H₂N-¹AVT.....⁵⁰K-OH) and the COOH-terminal domain (H₂N-⁵¹PGS.....⁹⁵Q-OH), we demonstrate that elafin's anti-elastase activity resides exclusively in the COOH-terminus. Several characteristics of elafin suggest potential anti-microbial activity. The anti-microbial activity of elafin, and of its two structural domains, was tested against the respiratory pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Elafin killed both bacteria efficiently, with 93% killing of *P. aeruginosa* by 2.5 µM elafin and 48% killing of *S. aureus* by 25 µM elafin. For both organisms, full-length elafin was required to optimise bacterial killing. These findings represent the first demonstration of co-existent anti-proteolytic and anti-microbial functions for elafin.

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Key words: Elafin; Human neutrophil elastase; Anti-microbial activity; *Pseudomonas aeruginosa*; *Staphylococcus aureus*

1. Introduction

As part of the immediate defence against bacterial pathogens, the lung is equipped with a number of endogenous anti-microbial peptides, including defensins, secretory leucocyte protease inhibitor (SLPI), lysozyme and lactoferrin [1–6]. In addition, bacteria stimulate the recruitment and activation of neutrophils, resulting in engulfment of organisms and ultimately their destruction by oxidants or proteolytic enzymes such as human neutrophil elastase (HNE) [7]. Natural inhibitors of HNE in the lung are thought to play a critical role in neutralising HNE liberated extracellularly, thus circumventing proteolytic damage to the host [8].

Elafin (also known as elafin-specific inhibitor (ESI) or skin anti-leucoprotease (SKALP)) is a low molecular weight (9.9 kDa) inhibitor of HNE and proteinase 3, which is secreted in the respiratory tract [9,10]. Along with α_1 -protease inhibitor (α_1 -PI) [8] and SLPI [11], elafin comprises an integral part of the 'anti-elastase shield' in the lung [12].

Several features suggest an additional anti-microbial function for elafin. In particular, elafin is highly cationic, it is

expressed selectively in the lung, the skin and at mucosal surfaces and SLPI (which has 42% sequence homology with elafin) has anti-microbial activity [5,13–17].

In this study, we describe an investigation of the anti-microbial activity of elafin against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, two important pulmonary pathogens with a particular propensity to antibiotic resistance [18–23].

2. Materials and methods

2.1. Proteins

Elafin peptides were produced synthetically in accordance with the derivation of amino acids from the established gene sequence [13] and were supplied by Albachem (Edinburgh, UK), using standardised protocols described elsewhere [24]. Three peptides were provided, namely full-length elafin (H₂N-¹AVT.....⁹⁵Q-OH), the NH₂-terminus domain (H₂N-¹AVT.....⁵⁰K-OH) and the COOH-terminus domain (H₂N-⁵¹PGS.....⁹⁵Q-OH). The terminal amino acid of the NH₂ domain and the first amino acid of the COOH domain were determined by analysis of the established crystal structure of a 57 amino acid fragment of elafin (H₂N-³⁹AQE.....⁹⁵Q-OH) [17], in conjunction with the established sequence of human SLPI, which has 42% sequence homology with elafin [13–15,25]. The molecular weights of elafin moieties, determined by mass spectrometry (Albachem, Edinburgh, UK) were 9925 Da for full-length elafin, 5172 Da for the NH₂-terminal domain and 4776 Da for the COOH-terminal domain.

Human serum albumin (HSA) was purchased from Sigma Chemicals (St. Louis, MO, USA). Recombinant human SLPI was purchased from R and D Systems (Minneapolis, MN, USA). Lyophilised preparations of elafin, SLPI and HSA were all reconstituted in 0.01 M K₂HPO₄/KH₂PO₄, pH 7.4 (phosphate buffer).

2.2. Bacteria

PAO1, a clinical strain and well-characterised type strain of *P. aeruginosa*, and C1705, a clinical strain of *S. aureus*, were available in-house [20,24].

2.3. HNE activity assay

The HNE activity assay has been described in detail elsewhere [26]. Briefly, serial dilutions of test inhibitor were added to 300 ng of purified HNE (Elastin Products, Owensville, MO, USA). All dilutions were performed in Tris 50 mM, Triton 0.1%, sodium chloride 0.5 M, pH 8.0. The samples were incubated for 15 min at 37°C before addition of the chromogenic substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (Sigma). In positive controls, buffer replaced test inhibitor. The change in absorbance, measured spectrophotometrically at 405 nm (MR5000 Plate Reader, Dynatech, Dynex, Billingham, UK), was expressed as a function of time. The inhibitory capacity of test inhibitors was derived by extrapolation to the ordinate of curves obtained by plotting the HNE activity against the concentration of inhibitor added, as described elsewhere [27].

2.4. Assay of microbial activity

Bacteria were grown initially as colonies on Columbia agar (Unipath, Basingstoke, UK) and, then, in 10 ml tryptone soya broth (TSB) (Unipath) overnight at 37°C in an orbital shaker (Gallenkamp, Fisher Scientific, Loughborough, UK) at 200 rev/min. 100 µl of the bacterial culture was resuspended in 10 ml fresh TSB and incubated for 3 h at 37°C with rotation, corresponding to a point compatible with logarithmic growth for both PAO1 and C1705. The resulting suspension

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Abbreviations: HNE, human neutrophil elastase; α_1 -PI, α_1 -protease inhibitor; SLPI, secretory leucocyte protease inhibitor; HSA, human serum albumin; TSB, tryptone soya broth; LPS, lipopolysaccharide; CF, cystic fibrosis

was centrifuged at 4500 rpm for 20 min at room temperature (Bio-fuge, Heraeus Instruments, Kendro, Bishops Stortford, UK), the supernatant discarded and replaced with 10 ml phosphate buffer and then resuspended. This process was repeated once. The quantity of viable bacteria was calculated from pre-constructed growth curves and dilutions made in phosphate buffer to give an estimated count of 5×10^4 viable colonies per ml.

In experiments using purified elafin, HSA or SLPI, 30 μ l aliquots of the bacterial suspension were added to 90 μ l aliquots of test substance, each diluted in phosphate buffer to give final concentrations of 1, 2.5, 10 or 25 μ M of the test substance. In positive controls, 90 μ l of phosphate buffer replaced the test solution and in negative controls, 30 μ l of phosphate buffer replaced the bacterial suspension.

The test bacterial suspension mix was incubated for 2 h at 37°C. Appropriate dilutions were made in phosphate buffer and 100 μ l aliquots plated out on Columbia agar. Colonies were counted after incubation of the plates for 16 h at 37°C.

2.5. Statistics

Data pertaining to *S. aureus* were normally distributed and comparisons between test substances and controls were performed using the paired *t*-test. Data pertaining to *P. aeruginosa* were not normally distributed and comparisons between test substances and controls were performed using the Wilcoxon signed rank test. Statistical significance was regarded as $P < 0.05$.

3. Results

3.1. Anti-elastase activity of elafin moieties

All three peptides (full-length elafin, the NH₂-terminal domain and the COOH-terminal domain) were tested for anti-HNE activity (Fig. 1). No anti-HNE activity was detected in the NH₂-terminal domain. In contrast, the COOH-terminal domain and the full-length molecule were found to have an identical anti-HNE activity, indicating that elafin's anti-elastase

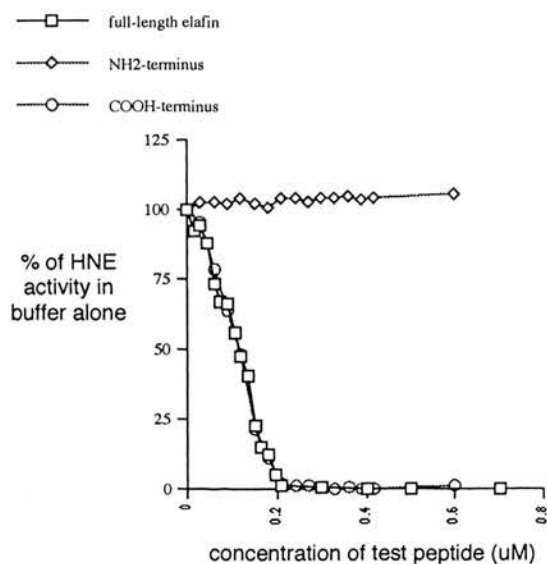


Fig. 1. Anti-elastase activity of full-length elafin, NH₂-terminal domain elafin and COOH-terminal domain elafin. Known quantities of test inhibitor were added to 300 ng HNE. All dilutions were performed in Tris 50 mM, Triton 0.1%, sodium chloride 0.5 M, pH 8.0. Chromogenic substrate (*N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide) was added and the change in absorbance at 405 nm was measured as a function of time. The molecular weights of full-length elafin, NH₂-terminal domain elafin, COOH-terminal domain elafin and HNE are 9.9, 5.2, 4.8 and 30 kDa, respectively. Results are expressed as a percentage of the HNE activity when incubated with buffer alone.

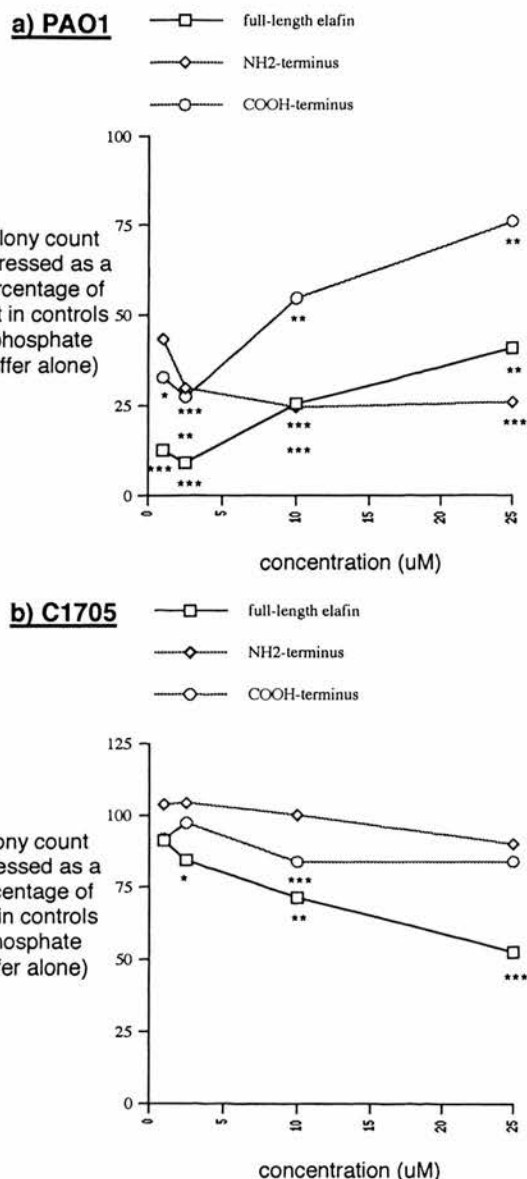


Fig. 2. Anti-microbial effect of elafin moieties against *P. aeruginosa* (PAO1) and *S. aureus* (C1705), expressed as a percentage of the colony count in phosphate buffer alone (control). In (a), results represent medians ($n = 8$ for concentrations of 2.5–25 μ M; $n = 4$ at 1 μ M). In (b), results represent means ($n = 5$). * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

tase activity resides exclusively in the COOH-terminal domain.

3.2. Anti-microbial activity of elafin

Full-length elafin resulted in a significant killing of PAO1 at all doses tested (1–25 μ M) (Fig. 2). The maximum effect was observed at 2.5 μ M, at which 93% of PAO1 was killed, relative to PAO1 grown in phosphate buffer alone. At 1 and 2.5 μ M, the contributions of the NH₂-terminus and COOH-terminus were approximately additive, but at higher doses, the predominant anti-microbial effect resided in the NH₂-terminal domain. The anti-microbial effect of full-length elafin against

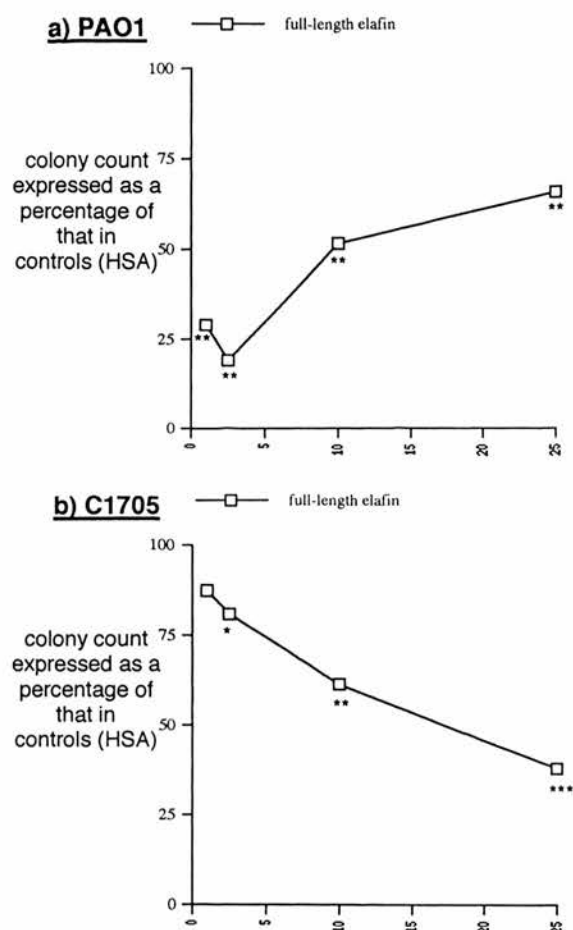


Fig. 3. Anti-microbial effect of full-length elafin against *P. aeruginosa* (PAO1) and *S. aureus* (C1705), expressed as a percentage of the colony count in HSA. In (a), results represent medians ($n=8$ for concentrations of 2.5–25 μM ; $n=4$ at 1 μM). In (b), results represent means ($n=5$). Comparisons were made between equimolar concentrations of test peptide and HSA. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. For reasons of visual clarity, only data for full-length elafin are shown, however, dose-response curves for NH_2 -terminal domain elafin and COOH-terminal domain elafin also closely paralleled those shown in Fig. 2.

S. aureus (C1705) was dose-dependent and was significant over a dose range of 2.5–25 μM (Fig. 2). At 25 μM , 48% killing of C1705 was achieved. The NH_2 -terminus showed a significant anti-microbial activity only at 25 μM and the COOH-terminus only at 10 μM . To ensure that the effects observed were not attributable to a non-specific peptide effect, the effects of full-length elafin, the NH_2 -terminal domain and the COOH-terminal domain against PAO1 and C1705 were also compared with a control protein (HSA) (Fig. 3). For full-length elafin, the maximal activity was described at 2.5 μM against PAO1 (81% killing) and at 25 μM against C1705 (63% killing) (Fig. 3), closely in keeping with the findings when the effect of full-length elafin was compared with that of phosphate buffer alone (Fig. 2). The dose-response curves generated for both NH_2 -terminal domain elafin and COOH-terminal domain elafin against PAO1 and C1705 also closely paralleled those obtained when survival was expressed relative to PO_4 buffer instead of HSA (data not shown).

3.3. Anti-microbial activity of SLPI

In a separate set of experiments, the activity of SLPI against PAO1 and C1705 was tested over the dose range 1–10 μM and compared with that of full-length elafin (Table 1). The anti-microbial activity of elafin against PAO1 conformed to the characteristic pattern shown in Fig. 2, with a maximal effect at 2.5 μM . The anti-microbial activity of elafin was greater than that of SLPI at all doses tested. To test for potential synergy, equimolar concentrations of SLPI and elafin (0.5 μM elafin added to 0.5 μM SLPI) were compared with either 1 μM elafin or 1 μM SLPI alone. No evidence of a synergistic effect was found (86% killing of PAO1 using the elafin/SLPI combination, 88% using elafin alone, 80% using SLPI alone). The anti-microbial activity of elafin and SLPI against C1705 was similar over the dose range tested.

4. Discussion

Elafin (ESI/SKALP) was originally characterised and sequenced from human bronchial secretions and from human psoriatic skin, on the basis of its anti-elastase activity [9,14]. The identification of anti-microbial activity against Gram-negative and Gram-positive respiratory pathogens at concentrations potentially achievable in epithelial lining fluid [28] (especially after genetic augmentation [26]) suggests more complex functions for elafin in the context of inflammation. This observation is in keeping with the identification of additional functions for other major anti-elastases such as SLPI, which has intrinsic anti-bacterial and anti-viral activity [5,16,29] and which can influence the function of lipopolysaccharide (LPS) [30] and prostaglandins [31].

Indeed, elafin can be added to the growing list of endogenous lung peptides which harbour an anti-microbial function, including defensins, SLPI, lactoferrin and lysozyme [1–6]. These molecules share certain general characteristics, for example, a low molecular weight and net positive charge. However, each has unique structural features, which may play a role in determining the anti-microbial function. The gene sequence and derived amino acid sequence of elafin have allowed identification of various structural determinants [13]. The NH_2 -terminal domain, as defined in this study, has a net positive charge of +5, as well as repeated structural motifs potentially acting as substrate for transglutaminase which may allow elafin to bind to the interstitium covalently [13,15,32]. The COOH-terminal domain, as defined in this study, has a net positive charge of +2 and has four disulfide bonds which confer structural stability [13,32]. Previous studies using elafin fragments of either 57 amino acids (H_2N -

Table 1
Anti-microbial effect of full-length elafin and SLPI against *P. aeruginosa* (PAO1) and *S. aureus* (C1705)

PAO1	1 μM	2.5 μM	10 μM
Full-length elafin	95.7	96.3	85.1
SLPI	93.8	94.7	73.1
C1705			
Full-length elafin	−9.8	−7.9	13.6
SLPI	−8.1	18.1	6.0

Results are expressed as percentage killing relative to that in PO_4 buffer alone (taken as 0%) and represent medians from three separate experiments.

When expressed relative to HSA, the results obtained were similar and followed the same trend (data not shown).

³⁹AQE.....⁹⁵Q-OH) or 48 amino acids (H₂N-⁴⁸STK.....⁹⁵Q-OH) have demonstrated high affinity anti-elastase activity (K_i in the nM range), but were unable to provide information concerning the NH₂-terminal end of the molecule [9,14,33]. These findings, in conjunction with crystallographic studies [17], suggested that elafin's anti-elastase activity resides exclusively in the COOH-terminal domain. Using discrete functional domains of elafin and studying full-length elafin for the first time, we have been able to provide direct confirmatory evidence to support this hypothesis by demonstrating that the COOH-terminal domain of elafin and the full-length molecule bind HNE with an equal efficiency (Fig. 1).

Furthermore, the use of discrete functional domains of elafin has allowed us to infer which regions of the molecule may be responsible for anti-microbial effects. In the case of *P. aeruginosa*, our data suggest that inherent anti-microbial activity exists in both structural domains and imply that their interaction may be necessary to augment anti-microbial activity at low concentrations. The inverse dose-response relationship noted may reflect the ability of *P. aeruginosa* to use higher concentrations of peptide as nutrients, promoting growth and competing with the killing effect of elafin. Indeed, *P. aeruginosa* is known to grow in increasing concentrations of organic nitrogen [34]. This may explain why elafin's anti-microbial effect was slightly less pronounced when albumin was used as control (see Fig. 3).

The anti-microbial effects of several low molecular weight peptides have been ascribed to their cationic nature. Cationic peptides are capable of binding *P. aeruginosa* LPS, altering the outer membrane stability and increasing susceptibility to other bactericidal compounds [35]. While the charge may have contributed to the killing observed here, it is unlikely to explain the entire anti-microbial effect of elafin (especially at low doses, where the less cationic COOH-terminal domain was more effective).

The anti-microbial activity against *P. aeruginosa* was independent of the anti-elastase activity of elafin, as the majority of anti-microbial activity resided in the NH₂-terminal domain, while anti-elastase activity resided exclusively in the COOH-terminal domain. Interestingly, a quite different, dose-dependent pattern of anti-microbial activity was seen against *S. aureus*. The NH₂-terminal domain alone contributed only slightly and at high concentrations. As with *P. aeruginosa*, this suggests that a simple charge effect is unlikely to explain elafin's anti-microbial effect. The observation that full-length elafin was more effective than the additive effects of the two structural domains may again imply a critical interaction between these. In a similar study using structural domains derived from SLPI, Hiemstra et al. [5] also found that the full-length molecule was more active against *Escherichia coli* and *S. aureus* than was either terminal fragment.

In our hands, SLPI was found to have anti-microbial activity against *P. aeruginosa* and *S. aureus* as has been described elsewhere [5,16]. The anti-microbial effects of SLPI were less pronounced in our study, but this may reflect the use of different clinical strains. Our data suggest that in equimolar concentrations, elafin is at least as effective as SLPI against PAO1.

The anti-microbial activity of elafin adds to the emerging picture of its primary role in the lung defence. Elafin is ideally placed to promote the early eradication of invading pathogens and to protect the host against proteolytic destruction in the

event of neutrophil recruitment. Indeed, the co-existence of anti-microbial and anti-elastase activity in the elafin molecule could have therapeutic implications. *P. aeruginosa* and *S. aureus* can each cause severe pneumonia [18,21] and frequently co-colonise patients with cystic fibrosis (CF) [19]. The significant morbidity and mortality associated with these organisms, coupled with their propensity to develop resistance to conventional antibiotics [22,23], demands the development of novel anti-microbial strategies. In CF, HNE is thought to contribute to the airway pathology by degrading substrate in the interstitium, enhancing inflammatory cell chemotaxis, stimulating mucous hypersecretion and promoting the adherence of *P. aeruginosa* [36–38]. Furthermore, PAO1 is known to promote significant release of elastase from hamster neutrophils in vivo [39]. Effective augmentation of anti-microbial anti-elastases for patients known to be at risk of developing infection with *P. aeruginosa* and/or *S. aureus* may thus be theoretically desirable. Our findings suggest that elafin gene augmentation could be particularly beneficial in CF. Elafin levels are known to be reduced in CF [40], high levels of elafin can be effected using adenoviral gene therapy in rats in vivo [26], elafin's transglutamination sites may confer a longer biological half-life in vivo [15,32] and elafin appears particularly active against the non-mucoid clinical isolate PAO1. Strategically, eradication of *P. aeruginosa* whilst still in the non-mucoid form may be especially important in preventing or delaying progression to chronic infection with mucoid variants, which are seldom eradicated and are associated with a significantly worse prognosis in CF [20].

We recently showed that genetic augmentation of elafin using adenoviral gene therapy protects human alveolar epithelial (A549) cells against HNE and activated neutrophils [41]. We have extended these findings to show that elafin's anti-microbial activity against PAO1 can be augmented in supernatant derived from A549 cells transfected with adenovirus encoding elafin (unpublished data). This suggests that genetic augmentation of endogenous anti-microbials may be effective against pulmonary pathogens, as has also been demonstrated using γ -interferon [42,43].

In summary, these findings demonstrate for the first time that elafin has an intrinsic anti-microbial activity against important Gram-negative and Gram-positive respiratory pathogens and that this activity is independent of the molecule's anti-elastase activity.

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